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Protection of healthy tissues from infection with systemically administered vaccinia virus strains

Dissertation

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Würzburg, January 2012

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1 Summary

Oncolytic virotherapy using recombinant vaccinia virus strains is a promising approach for the treatment of cancer. The generated virus strains appear to be very efficient in targeting and shrinking cancers that are resistant to chemo- and radiation therapy, while displaying relatively low side effects. The recombinant oncolytic vaccinia virus GLV-1h68 is presently in clinical trials for the treatment of human patients with different forms of cancer. This triple-mutant viral strain features strong oncolytic potential in cultures and in animals as well as in humans. To further improve the safety of oncolytic vaccinia viruses, the cellular microRNA machinery can be applied as the host's own security mechanism to avoid unwanted viral replication in healthy tissues.

MicroRNAs are a class of small single-stranded RNAs which due to their ability to mediate post-transcriptional gene-silencing, play a crucial role in almost every regulatory process in cellular metabolism. Gene silencing is mediated via mRNA degradation or translational repression depending on the complementarity of the microRNA seed region with its target mRNA. Different cancers display unique microRNA expression patterns, showing significant up- or downregulation of endogenously expressed microRNAs. Furthermore, the behavior of cancer cells can be altered by either adding microRNAs known to inhibit cancer cell spread and proliferation or suppressing cancer promoting microRNAs (oncomirs) making microRNAs promising targets for cancer gene therapy. The cell's own RNAi

machinery can also be utilized to control viral replication due to the virus dependence on the host cell replication machinery, a process controlled by microRNAs.

GLV-1h68 is a replication-competent vaccinia virus which carries three reporter genes for detection and attenuation purposes. Though there are hardly any side effects found in GLV-1h68 mediated oncolytic therapy an increased tropism for replication exclusively in cancer cells is desirable. Therefore it was investigated whether or not further cancer cell specificity of a recombinant vaccinia virus strain could be obtained without compromising its oncolytic activity using microRNA interference. To achieve this goal four copies of the mature human microRNA let-7a target sequence was cloned behind the stop codon in the 3'end of the vaccinia virus *D4R* gene, using a GLV-1h68 derivative, GLV-1h190, as parental strain yielding the new recombinant virus strain GLV-1h250. The *D4R* gene belongs to the group of early transcribed vaccinia genes and encodes an essential enzyme, uracil DNA glycosylase, which catalyzes the removal of uracil residues from double-stranded DNA. A defect in *D4R* prevents vaccinia virus from entering into the intermediate and late phase of replication, leading to an aborted virus replication.

Let-7a is a well characterized microRNA known to be expressed in high levels in healthy tissues and strongly downregulated in most cancers. After expression of the target sequence from the vaccinia virus genome, the endogenously expressed microRNA-let-7a should recognize its target structure within the viral mRNA transcript, thereby binding and degrading the viral mRNA which should lead to a strong inhibition of the virus replication in healthy cells. This effect was observed when using the virus GLV-1h250 in a virus replication study in human endometrial

regenerative cells (ERC) in comparison to its parental strains GLV-1h190 and GLV-1h68. GLV-1h250 displayed a 10-fold reduction in viral replication rate when compared with GLV-1h190 and GLV-1h68. This was observed independently whether a low multiplicity of infection (MOI) of 0.01 or a high MOI of 10 was used suggesting successful inhibition of viral replication in healthy cells. Furthermore, captured pictures of the cells under a fluorescent microscope at each time point during the experiment revealed that GLV-1h250 displayed less GFP expression within and showing an overall increased health status of the cells in which GLV-1h250 replicated in relation to GLV-1h68 and GLV-1h190 validating the titration data and emphasizing a decreased replication and lytic activity of the virus in healthy cells.

To investigate whether the replication efficiency of GLV-1h250 in cancer cells was comparable to GLV-1h68 which is used in clinical trials (GL-ONC1), virus replication studies were conducted using A549 lung carcinoma cells, a cell line known to show a strong downregulation of microRNA-let-7a. In this case, GLV-1h250 showed no significant inhibition of replication when compared to its parental strain and GLV-1h68 at a low MOI of 0.01 over a time course of 96 hours. These effects were observed by fluorescence microscopy where marker gene expression was monitored and virus titration of the harvested cells was conducted to investigate the presence of live virus particles. These findings indicated that the oncolytic activity of GLV-1h250 in cancer cells was not reduced in contrast to healthy cells.

To demonstrate the tumor specificity of GLV-1h250 *in vivo* A549 lung adenocarcinoma tumor bearing nude mice were injected with a high dose of viral particles (1×10^7 pfu / mouse) and monitored for seven weeks to investigate possible

toxic effects. Viral replication was monitored by observation of GFP expression under a UV light source. The tumor regression was measured using a caliper, the bodyweight and overall health condition of the animals were evaluated on a regular basis. The bodyweight was stable for most animals although the group injected retro-orbitally with the GLV-1h250 strain showed the highest average bodyweight with tumor volumes comparable to the GLV-1h68 and GLV-1h190 treatment groups. The mice treated with either GLV-1h68 or GLV-1h190 displayed animals with toxic side effects in form of pox formation on the body and tail. No virus was found in any of the organs in all mice treated with GLV-1h250 during the complete time course of the study in contrast to the other two tested strains, GLV-1h190 and GLV-1h68. GLV-1h250 was only present within the tumor. Overall, GLV-1h250 displayed a tumor regression potential comparable to GLV-1h68 and GLV-1h190 whereas replication took place exclusively in the tumor in contrast to its parental strain GLV-1h190 and GLV-1h68.

Taken together, this study provided a proof of concept that a microRNA regulated oncolytic vaccinia virus can selectively target cancer cells with reduced toxicity to healthy tissues. In addition, these findings suggest that other tissue specific microRNAs may be used for the targeting of vaccinia virus replication and that the same principle may be applied to not attenuated wild-type viruses that are considered promising candidates for oncolytic virotherapy but were found to be too toxic to healthy tissues.

Lastly, these experiments demonstrate the first step to assembling components of the nuclear splicing machinery in the cytoplasm of eukaryotic cells by successfully

expressing and processing the vaccinia virus synthesized target RNA by mature miRNAs of host origin.

Zusammenfassung

Die onkolytische Virotherapie mit rekombinanten Vaccinia Virusstämmen stellt einen vielversprechenden Ansatz zur Behandlung von Krebs dar. Die hergestellten Virusstämme scheinen effektiv Chemo- und Bestrahlungstherapie-resistente Arten von Krebs zu attackieren und zu reduzieren und zeigen dabei wenige Nebenwirkungen. Das rekombinante onkolytische Vaccinia Virus GLV-1h68 ist derzeit zur Evaluation in klinischen Studien für die Therapie von humanen Patienten mit diversen Formen von Krebs. Dieser dreifach mutierte Virusstamm zeigt sowoh in Zellkulturen wie auch in Tieren und Menschen starkes onkolytisches Potenzial. Um die Sicherheit von onkolytischen Vaccinia Viren zu erhöhen wird die zelluläre MikroRNA Maschinerie als körpereigener Abwehrmechanismus genutzt um ungewollte virale Replikation in gesundem Gewebe zu verhindern

MikroRNAs sind kurze, einzelsträngige RNA-Moleküle die aufgrund Ihrer Fähigkeit des Posttranscriptional Gene Silencing (RNA Interferenz) eine entscheidende Rolle in fast jedem regulativen Prozess im Zellmetabolismus spielen. Das Gene Silencing, vermittelt entweder durch mRNA Degradation oder translationale Repression, ist von dem Komplementaritätsgrad der MikroRNA und Ihrer Zielstruktur abhängig. Diverse Arten von Krebs zeigen spezifische MicroRNA-Expressionsmuster, welche sich als signifikante „Up“-oder „Down“-Regulation der Expression dieser microRNA(s) darstellt. Weiterhin kann das Verhalten von Krebszellen verändert werden, entweder durch Wiedereinbringen von in bestimmten Krebsarten „down“-regulierten

MikroRNAs oder durch Unterdrückung der Expression von MikroRNAs, die als krebsfördernd gelten (Oncomirs). Die RNA-Interferenz Maschinerie der Zelle kann des Weiteren auch als Replikationskontrolle z.B. von Viren genutzt werden, da Viren für Ihre eigene Vermehrung auf die Replikationsmaschinerie der Zelle angewiesen sind, ein Prozess welcher von MikroRNAs kontrolliert wird.

GLV-1h68 ist ein replikationskompetentes Vaccinia Virus, welches drei verschiedene Reportergene enthält, welche zu Erkennungs- und Attenuierungszwecken genutzt werden. Obwohl eine Behandlung mit GLV-1h68 kaum Nebenwirkungen zeigt, wäre eine Replikation des Virus ausschliesslich in Krebszellen wünschenswert. Aufgrund dessen wurde versucht ein rekombinantes Vaccinia Virus zu generieren welches, unter Zuhilfenahme der RNA Interferenzmaschinerie der Zelle, ohne Einbusse seiner onkolytischen Fähigkeit ausschliesslich in Krebszellen repliziert. Um dies zu erreichen wurden 4 Komplementärsequenzwiederholungen der humanen microRNA-let-7a der 3‘-UTR des Vaccinia Virus *D4R*-Gens folgend kloniert, wobei GLV-1h190, ein GLV-1h68 Derivat, als parentaler Virusstamm verwendet wurde. *D4R* gehört zu der Gruppe der frühen Gene von Vaccinia und kodiert ein essentielles Enzym, Uracil-DNA-Glykosylase, welches das Entfernen von Uracilresten aus doppelsträngiger DNA katalysiert. Ein Defekt im D4R Gen verhindert den Eintritt von Vaccinia Viren in die intermediäre und späte Phase der Replikation, was zu einem Abbruch der viralen Replication führt.

Let-7a ist eine gut charakterisierte MikroRNA die eine hohe Expression in gesunden Geweben und eine starke „Down“-Regulation in Krebszellen zeigt. Nach der Expression der MikroRNA-komplementären Sequenzen durch das Virusgenom sollte

die zellulär exprimierte let-7a MikroRNA Ihre Zielstruktur auf der viralen mRNA erkennen und diese degradieren. Dies sollte eine starke Hemmung der viralen Replikation in gesunden Zellen zur Folge haben.

Dieser Effekt konnte demonstriert werden, indem endometriale regenerative Zellen (ERC) mit dem neu generierten Virus GLV-1h250 infiziert und mit dem parentalen Virus GLV-1h190 und GLV-1h68 verglichen wurden. GLV-1h250 zeigte eine 10-fache Verringerung der Replikationsrate im Vergleich zu GLV-1h190 und GLV-1h68. Diese Beobachtung war sowohl unter Verwendung eines geringen „Multiplicity of Infection“ (MOI) von 0,01 sowie eines hohen MOI von 10 möglich. Dies könnte auf eine erfolgreiche Replikationshemmung in gesunden Zellen hinweisen. Desweiteren zeigten mit einem Fluoreszenzmikroskop aufgenommene Bilder zu jedem Zeitpunkt einen besseren Allgemeinzustand und eine verringerte GFP-Expression der GLV-1h250-infizierten Zellen im Vergleich zu GLV-1h68- und GLV-1h190-infizierten Zellen. Diese Resultate validieren die Titrationsdaten und betonen die verminderte Replikationsrate und lytische Aktivität des Virus in gesunden Zellen.

Um zu untersuchen, ob die Replikationseffizienz in Krebszellen mit dem in klinischen Studien verwendeten GLV-1h68 (GL-ONC1) vergleichbar ist, wurden virale Aufnahme- und Replikationsstudien unter Verwendung von A549 Lungenkarzinomzellen durchgeführt, welche eine starke „Down“-Regulation von MikroRNA let-7a aufweisen. In diesem Fall war unter Verwendung einer niedrigen MOI von 0,01 über einen Zeitraum von 96 Stunden kein signifikanter Unterschied in der Replikation zwischen GLV-1h68 und GLV-1h250 zu sehen. Dieses Resultat wurde durch Vergleich der GFP-Expression mittels Fluoreszenzmikroskopie

verifiziert. Weitere Bestätigung wurde durch Titration der geernteten Zellen erbracht, in welcher die Präsenz lebender viraler Partikel untersucht wurde. Dies lässt darauf schliessen dass die onkolytische Aktivität in Krebzellen im Vergleich zu gesunden Zellen nicht eingeschränkt ist.

Um die Tumorspezifität von GLV-1h250 *in vivo* zu testen wurden A549 Lungenkarzinom-tragende Nacktmäuse mit einer hohen Dosis Virus (1×10^7 pfu/Maus) infiziert und für sieben Wochen beobachtet, um potentiell auftretende Nebenwirkungen zu untersuchen. Die virale Replikationsrate konnte durch Überwachung der GFP-Expression unter einer UV-Lampe geprüft werden. Die Tumorregression wurde durch Messung mit Hilfe eines Messschiebers geprüft. Eine Messung des Körpergewichts sowie eine Evaluierung des Allgemeinzustandes wurden regelmässig durchgeführt. Das Körpergewicht blieb stabil für die meisten Tiere, jedoch zeigte die mit GLV-1h250 retroorbital injizierte Gruppe die Tiere mit dem höchsten durchschnittlichen Körpergewicht. Das Tumorvolumen war mit den GLV-1h68 und GLV-1h190 behandelten Gruppen vergleichbar. Mäuse welche mit GLV-1h68 oder GLV-1h190 behandelt wurden, zeigten toxische Nebenwirkungen in Form von Pockenformation auf Körper und Schwanz. Es wurden keine viralen Partikel in den Organen der mit GLV-1h250 behandelten Mäuse im Kontrast zu den beiden anderen Behandlungsgruppen während der gesamten Zeit der Studie gefunden. GLV-1h250 konnte ausschliesslich in den Tumoren detektiert werden. Insgesamt zeigte GLV-1h250 ein vergleichbares onkolytisches Potential wie GLV-1h68 und GLV-1h190, wobei sich die Replikation des Virus GLV-1h250 anders als der parentale Stamm GLV-1h190 und GLV-1h68 jedoch exklusiv auf den Tumor beschränkt.

Zusammenfassend konnte in dieser Studie der Machbarkeitsnachweis erbracht werden das ein MikroRNA reguliertes Vaccinia Virus selektiv Krebszellen attackieren kann und dabei einer reduzierte Toxizität in gesunden Geweben zeigt. Desweiteren suggerieren die Funde die Verwendung anderer gewebespezifische MikroRNAs zur Kontrolle der Replikation von Vaccinia Viren und das gleiches Prinzip auf nicht-attenuierte Wildtyp Viren, welche als potenzielle Kandidaten für die onkolytische Virotherapie angesehen jedoch zu hohe Toxizität in gesunden Geweben vorweisen, angewendet werden könnte.

Letztlich demonstrieren diese Experimente den ersten Schritt zur Fertigung von Komponenten der nukleären Splicingmaschinerie im Zytoplasma von eukaryontischen Zellen durch erfolgreiche Expression und Prozessierung von Vaccinia Virus synthetisierter mRNA durch wirtseigene MikroRNA.

3 Introduction

3.1 Cancer

Cancer is one of the leading causes of death among humans worldwide, accounting for 7.6 million deaths in 2008 according to the current World Health Organization cancer report with lung, stomach and liver cancer displaying the highest mortality rates. The classical treatments for cancers such as chemotherapy or radiation and surgery are rather unspecific and cause many side effects in patients creating the need for new therapeutic approaches in form of targeted therapy. Recently, a new class of therapeutics, oncolytic viruses, showed promising results when utilized to target cancer cells. Recombinant oncolytic vaccinia virus GLV-1h68, a green fluorescent protein expressing vaccinia virus generated by Genelux Corporation in San Diego, CA (USA), successfully targeted and lysed different types of cancer *in vivo* and was therefore enrolled in clinical trials where it is currently being evaluated for human application (Gentschev et al. 2010 and 2011, Yu YZ et al. 2009, Zhang et al. 2007, Yu YA et al. 2009). In order to treat patients suffering from cancer with a virus a high level of security is necessary and a fast and reliable detection method of the virus is desirable. In case of GLV-1h68, this was achieved through a triple mutation of the virus leading to an attenuation level suitable for application in humans. The insertion of reporter genes encoding for proteins such as gfp facilitate the location of the virus within the cells. However, to ensure an even better drug tolerance the body's own safety mechanisms can be used to force viral replication and subsequent cell lysis to take place solely to the tumorous tissue. Most types of

cancer display a significant expression pattern of small regulatory RNAs (microRNAs), creating potential targets to develop new drugs for cancer gene therapy. Endogenous microRNAs control a large portion of various processes within the cell via post transcriptional gene silencing. A mature microRNA recognizes target structures on mRNAs in the cytoplasm of the cell and binds them leading to degradation or translational inhibition of the mRNA. This strategy was applied to create the virus GLV-1h250. This virus was derived from a GLV-1h68 derivative, GLV-1h190, and carried four sequence repeats complementary to human microRNA-let7a, a microRNA known to be strongly expressed in healthy cells but downregulated in many types of cancer. Viral mRNA carrying a microRNA-let7a target structure should be degraded in cells with high microRNA-let7a expression leading to a decrease in overall viral titers whereas viral replication should remain strong in cancer cells leading to the lysis and regression of the tumorous tissue.

3.1.1 Cancer development

Cancer arises from a single cell, yet is not a single disease but rather a diverse group of diseases generally characterized by uncontrolled cell growth within the body. In a healthy organism cells divide, proliferate and die in a very controlled manner. When a cell becomes damaged or old it undergoes the process of apoptosis (programmed cell death). Cells with induced apoptosis shrink, develop bubble-like blebs on their surface and degrade the chromatin in their nucleus. Their mitochondria break down with the release of cytochrome c and the cells break into small membrane-wrapped fragments which, in mammalian cells, release ATP and UTP. These nucleotides bind to $P2Y_2$ receptors on wandering phagocytotic cells like macrophages and dendritic

cells thereby producing a “find-me” signal and attract them to the dying cells (Elliot et al. 2009). Furthermore, the phospholipid phosphatidylserine, which is normally located within the plasma membrane is exposed on the surface of the dying cell. This “eat me” signal is bound by phosphatidylserine receptors on the phagocytes which then engulf the cell fragments (Ravichandran et al. 2010). Subsequently, the phagocytic cells secret cytokines such as IL-10 and TGF-ß to inhibit inflammation. Apoptosis can be induced through the withdrawal of signals necessary for continued survival of cells such as growth factors for neurons or withdrawal of IL-2, an essential factor for the mitosis of lymphocytes, or through binding of death activator proteins such as TNF-α, TNF-ß (both TNF receptor)and Fas ligand (Fas receptor/CD95) to their specific receptors on the cell surface thereby inducing the apoptosis cascade. All cell functions are controlled by its genes. A mutation in the genes within the cell division (mitosis) or cell death (apoptosis) pathways can lead to uncontrolled cell division or promote survival of potentially harmful cells. The result of either or both of these events leads to an increasing cell mass forming a lump referred to as primary tumor. This tumor can either be benign, characterized by an elevated yet controllable cell growth rate and non-invasive behavior towards surrounding cells or malignant thereby invading and destroying adjacent and foreign tissues. Malignant tumors often release cells from the primary tumor into the blood stream or lymph through a process termed Epithelial-Mesenchymal Transition (EMT, Dasgupta et al. 2008). The process of EMT generates morphologically and functionally distinct cell types during embryogenesis; in the adult it occurs during wound healing, tissue regeneration, and organ fibrosis to generate repair-associated mesenchymal fibroblasts. When these circulating tumor cells find an adequate niche the process of EMT allows them to

attach to the vessel wall and penetrate through the vessel into the surrounding tissue to settle and form secondary tumors, a process termed metastasis.

3.1.2 Reasons for cancer formation

A dividing cell undergoes the process of mitosis. This cell division and the process of cell death are tightly controlled by various pathways including the RAS / RAF / MAPK and the p53 signalling pathways to control cell fate. These processes can be altered in cancerous cells due to point mutations within the genes controlling cell growth and death. Cancerous cells develop from healthy cells via malignant transformation. Initially, the cell's gene expression is being altered which may occur spontaneously or be brought on by a carcinogen. Carcinogens include many chemicals, tobacco, viruses, radiation, and sunlight. However, not all cells are equally susceptible to carcinogens. A genetic flaw in a cell may make it more susceptible. Even chronic physical irritation may make a cell more susceptible to carcinogens. The second step in the development of cancer is promotion. Unlike carcinogens, promoters do not cause cancer by themselves. Instead, promoters allow a cell that has undergone initiation to become cancerous. Promotion has no effect on cells that have not undergone initiation. Therefore, the combination of a susceptible cell and a carcinogen, are needed to cause cancer. Some carcinogens are sufficiently powerful to be able to cause cancer without the need for promotion. For example, ionizing radiation can cause various cancers, particularly sarcomas, leukemia, thyroid cancer, and breast cancer.

3.1.3 Common mutations in human lung cancers

Activating RAS point mutations are found in approximately one-third of all human malignancies (Bos J.L. et al. 1989). RAS encodes for a 21 kD monomeric membrane-localized G protein and functions as a molecular switch (Barbacid M. 1987). Every mammalian cell contains at least three distinct RAS proto-oncogenes encoding closely related, but distinct proteins. Activating mutations in the Ras proteins lead to constitutive signaling, thereby promoting cell proliferation and inhibiting apoptosis. Mutations in KRAS account for 90% of RAS mutations in lung adenocarcinomas, and approximately 97% of KRAS mutations in non-small cell lung cancer (NSCLC) involve codons 12 or 13 (Forbes S. et al. 2006). KRAS mutations are also frequently found in colorectal and pancreatic carcinomas (Bos JL. 1989) but rather uncommon in lung squamous cell carcinomas (Brose MS. et al. 2002, Suzuki Y. et al. 1990). HRAS mutations frequently occur in kidney, thyroid and bladder carcinomas. NRAS mutations are found in melanoma and hepatocellular carcinoma. Mutations in KRAS were shown to be necessary to maintain the viability of tumor cells in the absence as well as the presence of tumor suppressor genes (Fisher G.H. et al. 2001). Furthermore, KRAS mutations were shown to be involved in the resistance to treatment of cancer with EGFR inhibitors (Pao W. et al. 2005). EGFR is a member of the ErbB family of receptor tyrosine kinases. It is a survival and proliferation factor for a variety of tumor types. EGFR signals through the RAS/RAF/MEK/ERK pathway. Upon activation by extracellular ligands, EGFR dimerizes and autophosphorylates the tyrosine residues in the C-terminal domain. This leads to RAS GTPase complexation with RAF to phosphorylate and thereby activate two MAPK kinases which in turn phosphorylate ERK. Phosphorylation of multiple nuclear transcription

factors by activated ERK ultimately leads to DNA synthesis and cell proliferation (see fig. 1).

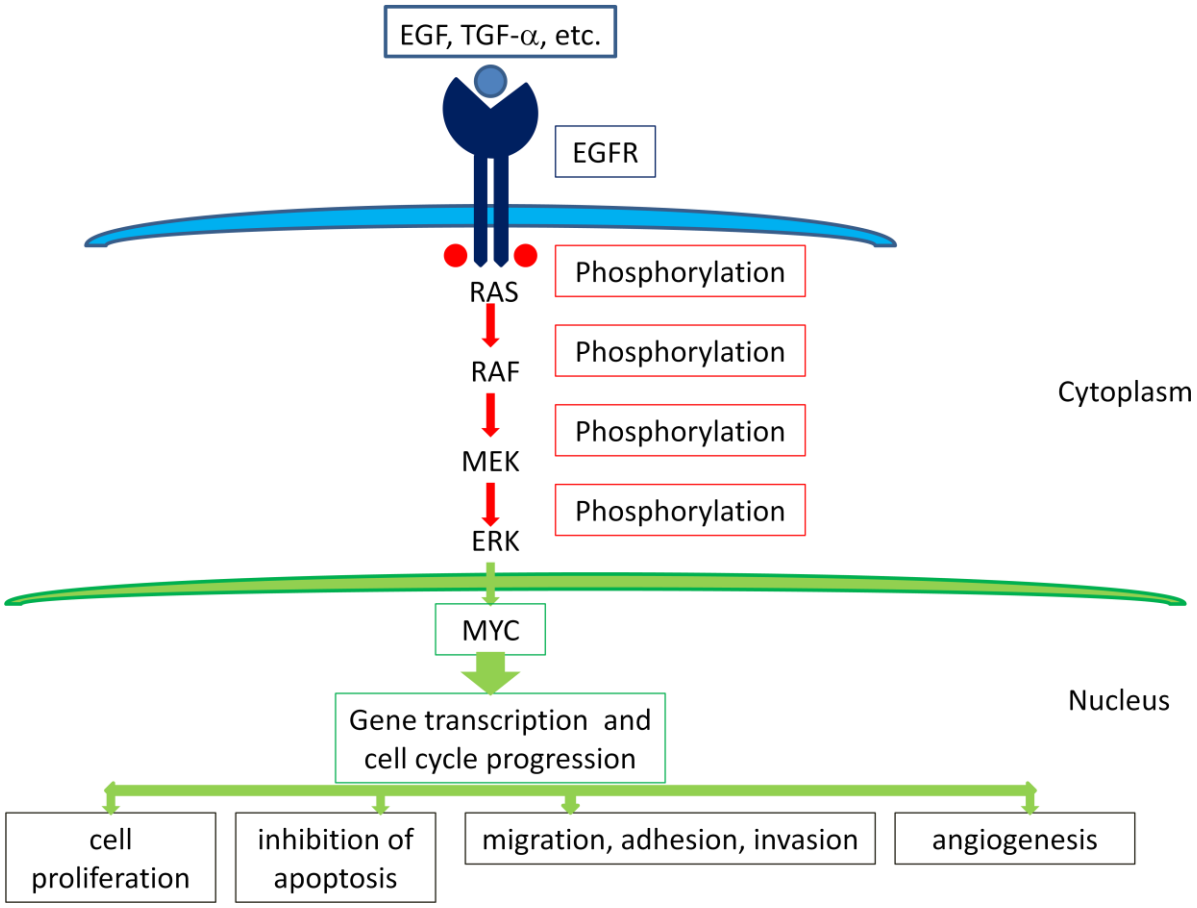


Figure 1: EGF receptor signaling pathway.

Upon activation by extracellular ligands, EGF receptor dimerizes and autophosphorylates. This leads to activation of RAS, which then complexes with RAF. The RAS/RAF complex then phosphorylates and thereby activates MEK. MEK in turn phosphorylates MAPK/ERK. Phosphorylation of multiple nuclear transcription factors by activated MAPK/ERK ultimately leads to DNA synthesis and cell proliferation. Constitutive activation of the RAS/RAF pathway can lead to uncontrolled cell growth via MYC activation.

The EGF signaling is stopped by protein phosphatases and the actions of Ras (GTPase activity). Tumors with mutations that constitutively activate RAS/RAF/MEK/ERK-pathway downstream EGFR can bypass the block by EGFR inhibitors, leading to drug resistance. RAS and MYC are well known to cooperate to

induce tumorigenesis. MYC is an oncogenic transcription factor that promotes tumorigenesis. The MYC family of proto-oncogenes encodes transcription factors that play a pivotal role in regulating cellular proliferation, cellular growth, differentiation, angiogenesis, adhesion, and apoptosis (Pelengaris S. 2002, Fachini LM. 1998, Dang CV. 1999, Sears 2002). Each member of the MYC family has been shown to be associated with specific types of human cancer, e.g. C-MYC translocation in a lymphoma is used as a biomarker in the identification of Burkitt's lymphoma. N-MYC amplification is associated with neuroblastoma, and L-MYC amplification is associated with small cell lung carcinoma (Nesbit CE. 1999). MYC is over expressed in almost half of human cancers, most commonly through epigenetic mechanisms. A critical feature of the regulation of the MYC protein is its phosphorylation to become activated and stabilized. The phosphorylation is regulated through AKT, and ERK and RAS (Sears R. 2000). A dominant negative form of MYC (Omomyc) was shown to induce cell death of KRAS-mutated human lung adenocarcinoma A549 cells *in vitro* and *in vivo* (Fukuzawa et al. 2010). These observations suggest that one way to target MYC for the treatment of cancer would be to target the inactivation of the gene products that regulate MYC phosphorylation.

HMGA2, a chromatin-binding protein, functions in transcriptional regulation and recent studies suggest a role in cellular senescence (DiCello et al. 2008). HMGA1 proteins also appear to participate in cell cycle regulation and malignant transformation, whereas HMGA2 has been implicated primarily in the pathogenesis of benign, mesenchymal tumors. It was shown that overexpression of HMGA2 lead to a transformed phenotype in cultured lung cells derived from healthy tissue (DiCello et al. 2008). It was found to be over expressed in NSCLC and inhibiting HMGA2 expression blocked the transformed phenotype in metastatic NSCLC cells (Meyer B.

et al. 2007). HMGA2 mRNA and protein were over expressed in primary human lung cancers compared with healthy tissue or indolent tumors indicating that HMGA2 is an oncogene important in the pathogenesis of human lung cancer (DiCello et al. 2008).

3.2 MicroRNAs

Recently, research interest has strongly increased in a class of small non-coding RNA species, known as microRNAs (miRNAs), which have critical regulatory functions in various biological processes (Bartel DP 2004, Ambros V. 2004). The identification and characterization of miRNAs is a rapidly growing area of research as miRNAs regulate a variety of processes such as development, cell proliferation and death, and have been recently linked to oncogenesis (Zhao et al. 2005, Yekta et al. 2004, Poy et al. 2004). MiRNAs are small endogenous RNA molecules of ~21-25 nucleotides in length that regulate gene expression by targeting one or more mRNAs for translational repression or cleavage. In 1993, Lee, Feinbaum, and Ambros discovered that lin-4 in *Caenorhabditis elegans (C. elegans)* did not code for a protein but instead produced a pair of short RNA transcripts that each regulated the timing of larval development by translational repression of lin-14, which encodes for a nuclear protein. They postulated the regulation was due to sequence complementarity between lin-4 and unique repeats within the 3' UTR of the lin-14 mRNA. A few years later the second miRNA, let-7, was discovered (Reinhart et al. 2000). The let-7 miRNA, similar to lin-4, also regulated developmental timing in *C. elegans*. Since the discovery of let-7, thousands of miRNAs have been identified in organisms as diverse as viruses, worms, and primates through random cloning and

sequencing or computational prediction (Rhoades et al. 2002, Enright et al. 2003, Krek et al. 2005, Kiriakidou et al. 2004).

3.2.1 MicroRNA biogenesis

The study of miRNA function by the suppression of miRNA expression in cells is a rapidly expanding area of research due to the thousands of microRNAs that have been identified in plants, animals, and viruses (Lai et al. 2002 and 2005, Lim et al. 2003 and 2005). MicroRNA genes reside in the genome as distinct transcriptional units as well as in clusters of polycistronic units with the information of several microRNAs (Lagos-Quintana et al. 2001, Lau et al. 2001, Lee et al. 2002, Reinhart et al. 2002). Studies suggest that approximately half of known microRNA reside in non-protein coding RNAs (intron and extron) or within the intron of protein coding genes (Rodriquez et al. 2004). MiRNAs are double-stranded RNAs of 21-25 nt that are derived from endogenously expressed transcripts with characteristic hairpin structures. The miRNA pathway (see fig. 2) begins with the transcription of a primary miRNA (pri-miRNA) from a miRNA gene. RNA polymerase II transcribes miRNA genes, generating long primary transcripts (pri-miRNAs) (Kim 2005). The pri-miRNAs are processed in the nucleus by the RNase-III enzyme Drosha and double-stranded RNA-binding domain (dsRBD) proteins yielding a hairpin precursors (pre-miRNA) consisting of approximately 70 nt. Once the pre-miRNAs are transported into the cytoplasm by exportin 5 in a Ran GTP-dependent mechanism, a RNase III Dicer, digests the pre-miRNAs resulting in a 21-25 nt unstable miRNA duplex structures (Sontheimer 2005). The less stable of the two strands in the duplex is incorporated into a multiple-protein nuclease complex, the RNA-induced silencing complex (RISC),

which regulates protein expression by aligning with the mRNA. The level of complementarity between the miRNA and its target sequence determines the mode of repression. Partial complementarity leads to translational repression and full complementarity leads to cleavage of the target mRNA (He et al. 2004, Pasquinelli et al. 2005). Research suggests that as many as one-third of human genes may be regulated by miRNAs (Lim 2003, Lim 2003). This occurs when mature miRNAs become coupled with a multiple-protein nuclease complex called the RNA-induced silencing complex (RISC). Once incorporated into a RISC, the miRNA is situated to regulate the target genes by degradation of the mRNA through direct cleavage or by inhibiting protein synthesis.

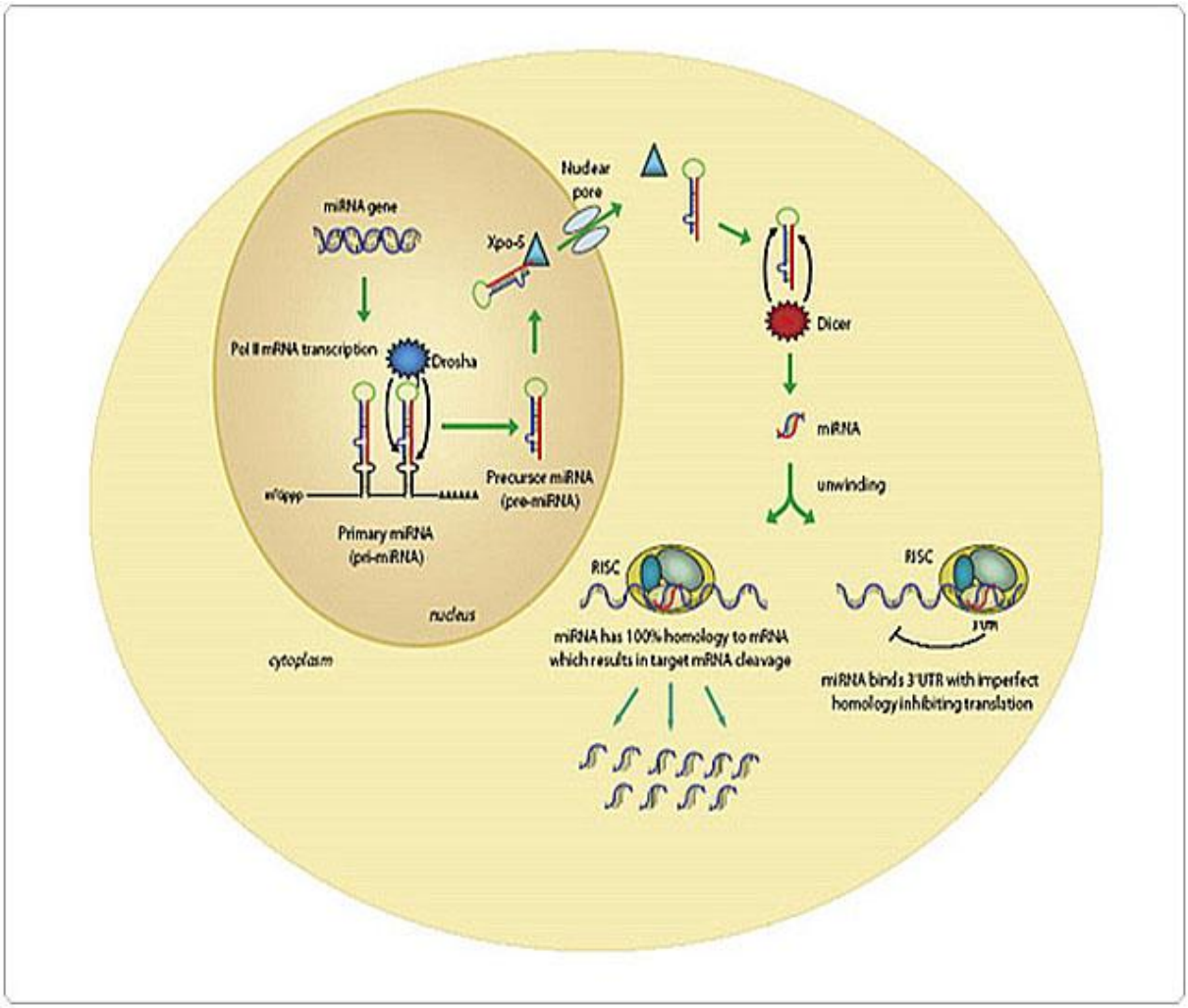


Figure 2. miRNA pathway in mammalian cells (from Marek Mraz, 2006).

1) miRNA gene is transcribed into a primary miRNA transcript (pri-miRNA) 2) Pri-miRNA is cleaved by Drosha to a hairpin pre-miRNAs 3) Pre-miRNA is transported out of the nucleus by exportin-5 4) Pre-miRNA is cleaved by Dicer to form a short double-stranded miRNA duplex 5) miRNA duplex separates into single-stranded mature miRNAs and complexes with a RISC-like structure 6) mRNA binds with miRNA/RISC complex 7) mRNA is translationally repressed.

3.2.2 MicroRNA-let7 and its role as tumor suppressor

The first identified miRNAs, the products of the *C. elegans* genes lin-4 and let-7, showed important roles in controlling developmental timing by regulating mRNA translation (Ambros et al. 1984, Lee et al. 1993, Reinhart et al. 2000). When lin-4 or let-7 was inactivated, specific epithelial cells underwent additional cell divisions instead of their normal differentiation. Because abnormal cell proliferation is a hallmark of human cancers, it seemed possible that miRNA expression patterns might denote the malignant state. Indeed, altered expression of a few miRNAs has been found in some tumor types (Michael et al. 2003, Calin et al. 2002, Eis et al. 2005, Johnson et al. 2005) and used as diagnostic tool in the characterization of various tumors. Lu et al. (2005) used a bead-based flow cytometric miRNA expression profiling method to present a systematic expression analysis of 217 mammalian miRNAs from 334 samples, including multiple human cancers. The miRNA profiles reflected the developmental lineage and differentiation state of the tumors. A general downregulation of miRNAs in tumors compared with healthy tissues could be observed. Furthermore, poorly differentiated tumors could be classified using miRNA expression profiles highlighting the potential of miRNA profiling in cancer diagnosis. Using genetic approaches and reporter constructs, Johnson et al. showed (2005) that the expression of *let-60/RAS* is regulated by two *let-7* miRNA family members (*let-7* and *miR-84*) via the 3'UTR of the *let-60/RAS*

gene. *let-60/RAS* is the *C. elegans* orthologue of the human RAS genes, HRAS, KRAS, and NRAS. All three human RAS 3'UTRs contain multiple putative *let-7* complementary sites with features of validated *C. elegans* Let-7 complementary sequence. Many of the Let-7 complementary sequences are conserved in rodents, amphibians, and fish, suggesting functional relevance. The presence of putative Let-7 complementary sequences in human RAS 3'UTRs suggests that mammalian *let-7* family members may regulate human RAS in a manner similar to the way *let-7* and *miR-84* regulate *let-60/RAS* in *C. elegans*. Takamizawa et al. (2004) evaluated the miRNA expression in lung cancer patients with stage IB or IIA squamous cell carcinoma using microarray analysis. The lung tumor samples displayed more than 50% reduction in levels of miRNA-*let-7* relative to the healthy adjacent tissues from the same patients. Only sporadic reduction in *let-7* was detected in breast and colon cancer samples. In addition, several human *let-7* family members have been mapped to chromosomal intervals that are deleted in lung cancers (Calin et al. 2004), which could explain the reduced *let-7* expression observed.

The two other oncogenes discussed earlier for playing an important in cancer development, MYC and HMGA2, are also regulated by miRNA-let7 (Mayr et al. 2007; Sampson et al. 2007) as well as cyclin-dependent kinases that contribute to the development of cancer (Johnson et al. 2007). Since many of these genes function in various pathways with altered regulation of expression in cancer, *let-7* is able to interfere with multiple cancer-associated pathways, such as mitotic signaling, cell cycle progression and angiogenesis. However, the suppression of *let-7* was found in all three major forms of NSCLC: adenocarcinomas, squamous and large cell carcinoma (Johnson et al. 2005) emphasizing the importance of microRNA-let-7 expression in lung cancer development. NSCLC is the most frequent human lung

cancer and the major cause of death from cancer in man. 50% of human NSCLC carry mutations in genes that encode activators of the mitogenic cascade (RAS-RAF-MEK-ERK) (Marks et al. 2007). This cascade is known to induce a target gene of ß-catenin and C-MYC (Kerkhoff et al. 1998). MYC is an evolutionarily conserved nuclear protein involved in the control of cell proliferation and differentiation (Luscher et al. 1990). In healthy cells, MYC protein levels are transiently elevated during cell growth but decline to low levels as cells exit the cell cycle (Persson et al. 1984, Eilers et al. 1991). The MYC oncogene, first described in 1982 (Vennstrom et al.), was recognized as a key transforming oncogenic agent in a wide variety of human tumors including Burkitt lymphoma, breast and cervical carcinomas, glioblastomas, and osteosarcomas (Facchini et al. 1998) and recently found to play an important role in NSCLC development (Allen et al. 2011). RAF/MYC cooperation in a murine hematopoietic system (Rapp et al. 2007 and 2008) displayed an effect on reprogramming events. In humans, amplification and rearrangements of MYC genes were found in a fraction of NSCLC (Yokota et al. 1988). Endogenous C-MYC was found to be involved in non metastatic KRAS-induced NSCLC (Soucek et al. 2007). The inducible expression or function of transgenic C-MYC demonstrated cooperation with mutant KRAS in tumor progression to various degrees (Tran et al. 2008, Murphy et al. 2008). Constitutive or inducible expression of C-MYC in addition to C-RAF in NSCLC cells was sufficient to rapidly induce metastasis to liver and lymph nodes (Rapp et al. 2009). The combination of C-MYC and C-RAF transgenes caused appearance of a phenotypic switch from cuboidal to alveolar papillary/columnar epithelial cells (APECs), known to be the most rapidly growing tumor cells and also predominate in liver metastasis. C-MYC induced production of VEGF by tumor cells leading to tumor vascularisation (Rawlins et al. 2008) demonstrating its impact on

cancer development and the importance of its controlled mediated via microRNA-let7 repression. Let-7 represses C-MYC by regulating the RNA-binding protein HuR. RNA-binding proteins (RBPs) are potent post-transcriptional regulators of gene expression, acting primarily through 5' and 3' untranslated regions (UTRs) (Ambros 2004; Moore 2005; Keene 2007; Filipowicz et al. 2008; Bartel 2009). The RBP HuR (human antigen R) stabilizes and modulates the translation of numerous target mRNAs (Hinman and Lou 2008). HuR was found predominantly nuclear but its post-transcriptional actions were linked to its cytoplasmic levels and its association with target mRNAs. These two processes were influenced by post-translational modification of HuR (Hinman and Lou 2008; Kim and Gorospe 2008). Through its actions on target mRNAs, HuR has been implicated in various biological processes, including cell division, immune and stress responses, differentiation, and carcinogenesis (López de Silanes et al. 2005; Hinman and Lou 2008; Kim and Gorospe 2008). The mRNA encoding the proto-oncogene C-MYC was identified previously as a target of HuR (Lafon et al. 1998). Kim et al. (2009) revealed that HuR bound the C-MYC 3'UTR at a site proximal to that bound by the miRNA let-7. However, analysis of endogenous C-MYC mRNA and C-MYC (3'UTR) reporter constructs showed that let-7 was required for HuR to repress C-MYC demonstrating the complexity of microRNA mediated translational repression.

HMGA2 codes for a small, nonhistone, chromatin-associated protein that can modulate transcription by altering the chromatin architecture but has no intrinsic transcriptional activity (Sgarra et al. 2004, Fedele et al. 2002). HMGA2 is primarily expressed in undifferentiated proliferating cells during embryogenesis and in a wide variety of benign and malignant tumors (Schoenmakers et al. 1995, Geurts et al. 1997, Mine et al. 2001, Fedele et al. 2001). In many of these tumors, a chromosomal

translocation truncates the human HMGA2 open reading frame (ORF), thereby retaining the three DNA-binding domains of HMGA2 while replacing the spacer and the acidic domain at the C terminal region (Schoenmakers et al. 1995 and 1999, Geurts et al. 1997, Mine et al. 2001, Petit et al. 1996). The loss of the C-terminal region is assumed to be the cause of oncogenic transformation. The translocations also replace the 3′ untranslated region (3′ UTR), leading to the assumption that transformation might be caused by the loss of repressive elements in the UTRs (Borrmann et al. 2001). It could be demonstrated that chromosomal rearrangements in some tumors left the ORF intact but disrupted the 3′ UTR which was associated with over-expression of the wild-type HMGA2 protein (Schoenmakers et al. 1995, Geurts et al. 1997, Inoue et al. 2006). The HMGA2 3′ UTR has seven conserved sites complementary to the *let-7* RNA (Lewis et al. 2005) indicating that the loss of *let-7* mediated repression might be a major mechanism of oncogenic HMGA2 translocations associated with various human tumors. Some of microRNA-let7's repressive functions in cancer development are summarized in figure3.

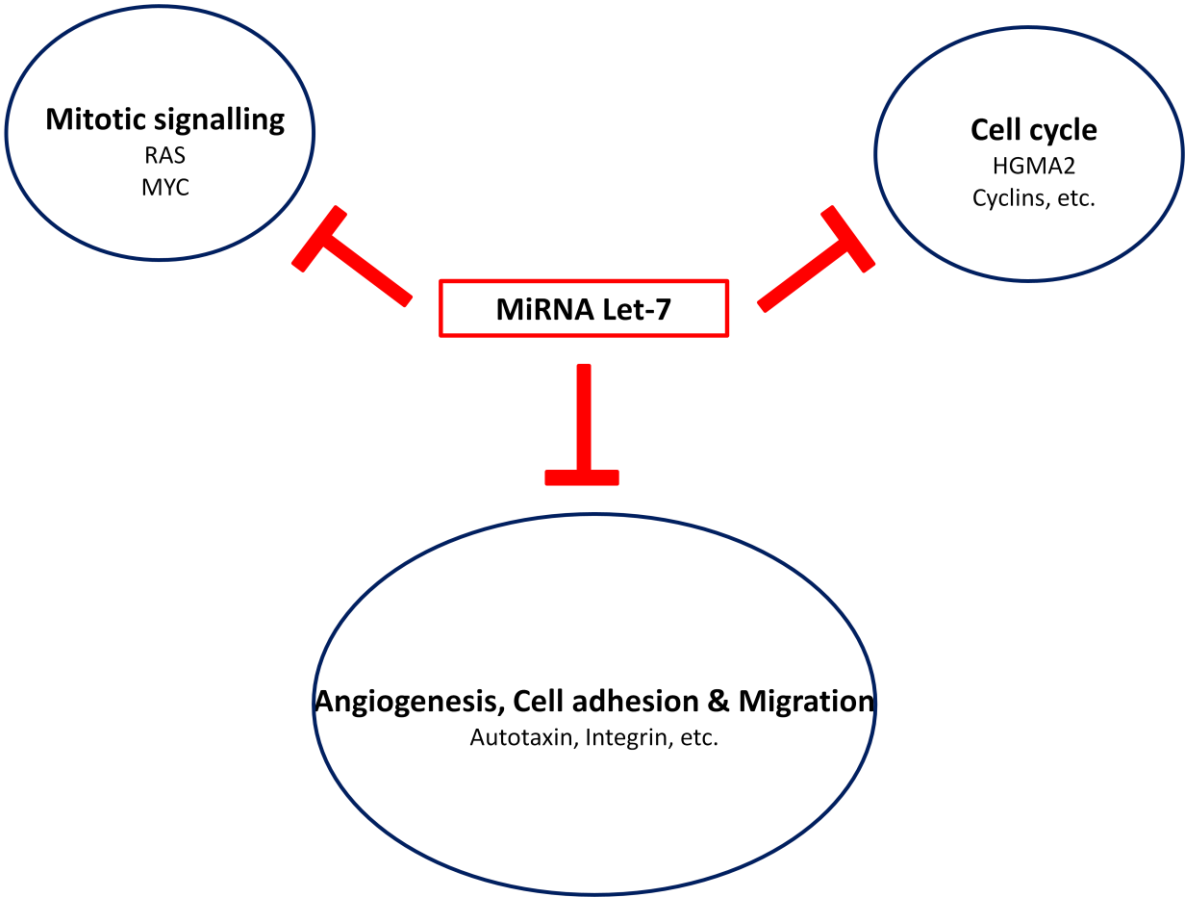


Figure 3: Targets of miRNA-let-7 involved in cancer development

miRNA-let7 represses mRNAs of mitotic signalling, cell cycle, angiogenesis, cell adhesion and migration.

Constitutive expression or mutation of oncogenes like RAS, MYC and HGMA2 are associated with human cancer (Reinhart et al. 2000, Malumbres et al. 2003). The observations that these genes are oncogenes, *let-7* is down-regulated in lung tumors, and their expression is regulated by *let-7* miRNA suggest that reduced *let-7* in lung tissue leads to over-expression of oncogenes and subsequently increased cell proliferation. The concept of miRNA Replacement Therapy is more and more used in cancer therapy (Bader et al. 2010). To achieve this, an endogenous miRNA that has been depleted in cancer is being added to the cells to reverse their uncontrolled proliferation due to insufficient amounts of endogenous microRNA expression levels. The finding of strongly down-regulated miRNA let-7 in cancer cells can also be used

to control the replication of oncolytic viruses and therefore more precise targeting of cancers by vaccinia virus-mediated oncolytic virotherapy.

3.3 Oncolytic virotherapy

Oncolytic viruses (OVs) are a promising and relatively new class of antitumor therapy undergoing increased clinical testing and application. Three OV platforms (vaccinia, HSV and Reo) are in or entering phase III clinical studies (clinicaltrials.gov). The clinical relevance of OVs is based on their capacity to preferentially replicate in and lyse cancer cells. They act both by direct destruction of tumor cells following specific replication and by delivery of established antitumor genes expressed by the engineered vectors when genetically modified. OVs can also stimulate or accentuate antitumor immune responses. Although efficacy of OVs has been established in animal models, efficacy in human trials has been much more limited, particularly when used as monotherapy due to very early clearance of the virus from the patient. Strategies to enhance the efficacy of OVs include enhancing tumor cell killing specificity and potency and by using such agents in combination with established antitumor therapies such as radiation or chemotherapy (Pandha et al. 2009). Clinical reports have described the regression of leukemia (Pasquinucci et al. 1971, Gross et al. 1971), Hodgkin's disease (Zygiert et al. 1971, Taqi et al. 1981) and Burkitt's lymphoma (Bluming et al. 1971) concomitantly with measles infection. Remissions also coincided with other naturally acquired viral infections, such as hepatitis, glandular fever, and measles (Taylor et al. 1953, Still et al. 1897). The foundation for the concept of oncolytic virotherapy came from the observation of Levaditi et al. in 1922 that a smallpox vaccine (vaccinia virus) was able to inhibit various tumors in

rats and mice. Pack reported in 1950 that he had observed a long remission of metastatic melanoma in a patient who was vaccinated against rabies after a dog bite. It was recognized early that viruses were capable of adapting themselves for replication in specific tissues. This property was applied to create a more oncolytic or more tumor-specific virus and by 1952 Moore could utilize the adaptive capacity of viruses to enhance oncolytic activity (Southam 1960, Moore et al. 1951, Hammon et al. 1963, Yohn et al. 1968). Even before recombinant DNA techniques became available, it was suggested that the alteration of the viral genome could provide improved targeting of oncolytic viruses (Southam 1960). It was first demonstrated in 1968 that genetic alteration of a viral genome was possible when polynucleotides were added to the tobacco mosaic virus genome (Rogers et al. 1968) leading to expression of polylysine. Subsequently, Southam et al. (1960) suggested the possibility of viral attenuation by genomic alteration of an oncolytic virus and the first studies took place in which an engineered virus was employed for cancer therapy. Ever since, the concept of oncolytic virotherapy was applied using vectors derived from viruses including measles, reovirus, vaccinia, herpes virus, and adenovirus, along with several newcomers, such as Seneca Valley virus and Coxsackie virus A21.

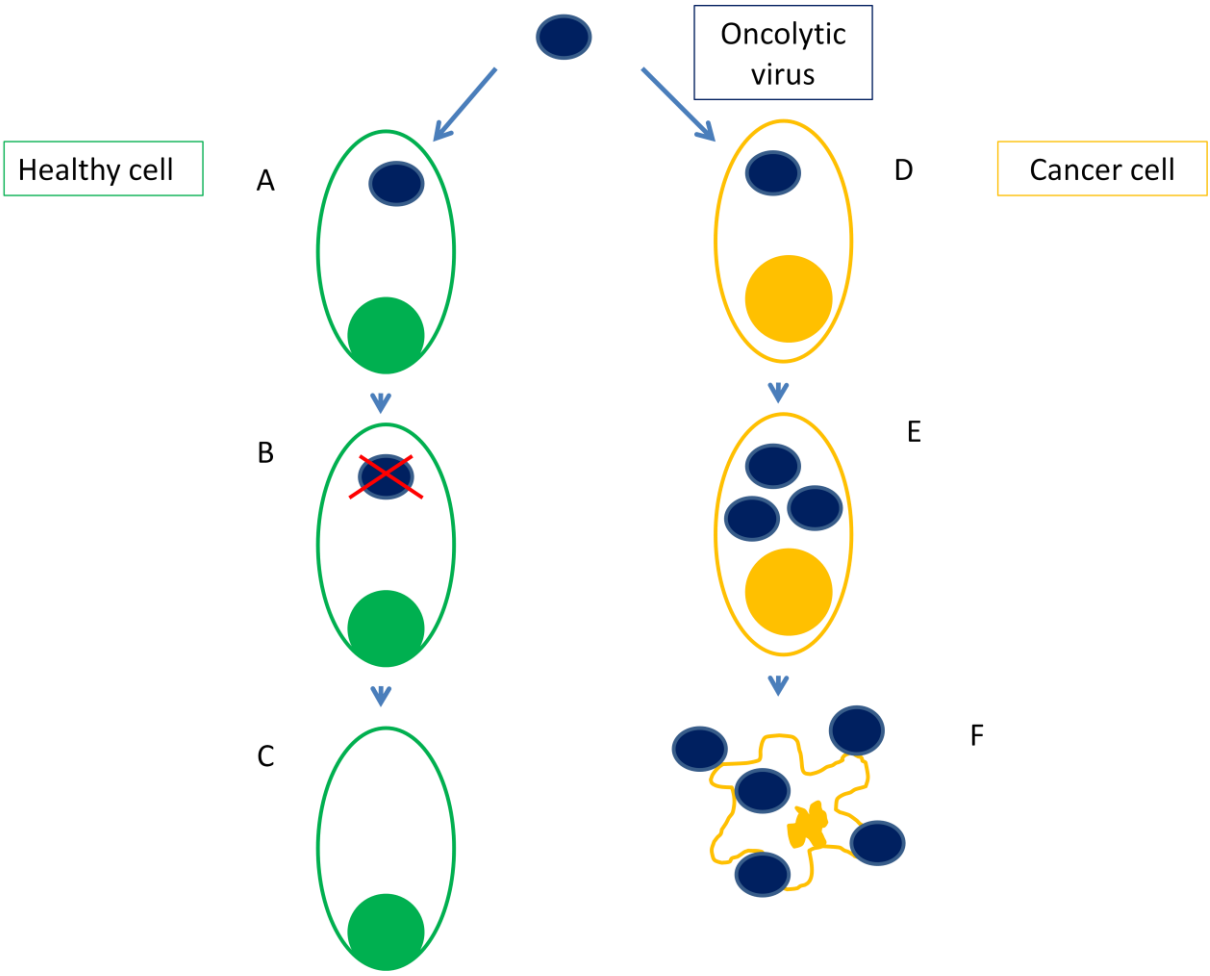


Figure 4 (A-F): Oncolytic virotherapy using vaccinia virus

Mode of action of oncolytic virus in cells. A-C: an oncolytic vaccinia virus enters a healthy cell where its replication is blocked and the healthy cell can proceed without lysis. D-F: the oncolytic vaccinia virus enters the cell and replicates due to the specific tumor cell milieu leading to lysis of the tumorous cell and viral spread.

Oncolytic therapy using recombinant vaccinia virus GLV-1h68 and its derivatives has various advantages when compared to other oncolytic viruses. Although vaccinia virus replicates in the cytoplasm, no viral DNA enters the nucleus and no viral DNA is integrated into the host genome, an issue that has to be considered when using nucleic replicating oncolytic viruses. The clinical relevance of GLV-1h68 could recently be demonstrated in a clinical trial setting with humans bearing various solid tumors. Vitell-Pedersen et al. 2011 could show that the administration of GL-ONC1 (resembling GLV-1h68) was well tolerated with minimal toxicity and preliminary

evidence of anti-tumor activity emphasizing the importance of vaccinia virus as an oncolytic agent.

3.3.1 Vaccinia virus life cycle

Vaccinia virus belongs to the family of poxviruses (Ryan et al.2004). Poxviruses are the largest known DNA viruses and are distinguished from other viruses by their ability to replicate entirely in the cytoplasm of the cells they colonize and replicate in. Infectious viral particles contain many of the enzymes needed for replication within its virion resulting in the large size of the virus. Because of its size, vaccinia was the first animal virus observed using microscopy. Specific enzymes, including DNA-dependent RNA polymerase, polyA polymerase, and several capping enzymes are all packaged within the core of the virus. The core also contains a 200-kilobase (kb), double-stranded DNA genome and is surrounded by a lipoprotein core membrane. The life cycle of vaccinia begins when the virus fuses with the plasma membrane of a susceptible cell via a protein-based entry-fusion complex or is absorbed by cellular endosomes. Once the virus has entered the cell, the viral core is released into the cytoplasm of the cell, where virally packaged transcriptases initiate transcription of early genes (see fig. 5). Only minutes after virus uptake into the cell, functional capped and polyadenylated mRNAs are produced and polypeptide synthesis begins. The initial proteins synthesized are used to uncoat the virus and to begin the process of viral DNA replication. The early genes code for factors that initiate the transcription of late genes. The late genes function in virion construction. Once virions are constructed and their DNA is capsulated, the virions acquire an envelope in the Golgi apparatus and are released from the cell by exocytosis as extracellular enveloped

virus (EEV) particles. The cell undergoes lysis 7-24 hours after initial virus uptake, releasing nonprocessed virions, which are visible under electron microscopy as intracellular naked virus (INV) particles. Despite the differences between EEV and INV particles, both forms are infectious. Each cell in which vaccinia virus replicates yields approximately 10,000 new viral particles.

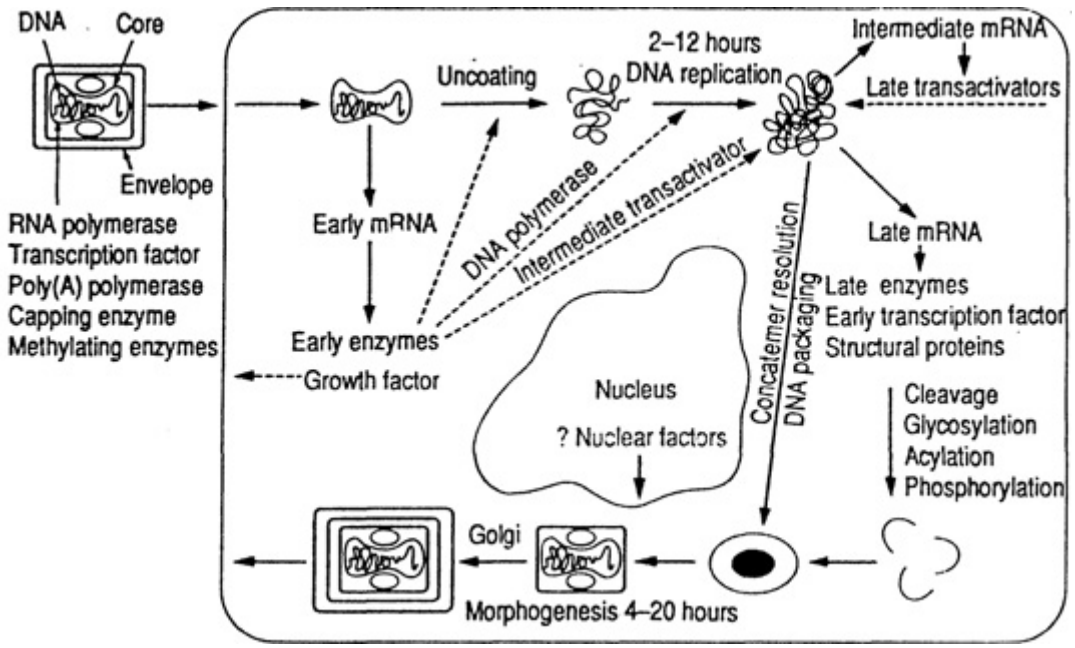


Figure 5: Vaccinia virus life cycle (from B. Moss, 1991)

After entering the cell the viral DNA is released into the cytoplasm where early genes are transcribed, followed by intermediate and late gene expression. After morphogenesis has taken place the mature enveloped viral particles lyse the cell wall and spread.

Since vaccinia virus has been studied extensively for more than a century, little is unknown about this virus making it a great candidate for safe oncolytic virotherapy in human patients with cancer. However, due to its aggressive nature the recombinant vaccinia virus GLV-1h68 was mutated three times to ensure an attenuation level suitable for human application. GLV-1h68 is a replication-competent engineered

vaccinia virus which has been attenuated by inserting reporter genes into loci responsible for the virulence of vaccinia virus (Zhang et al. 2007). The transgenes encoding *Renilla* luciferase, green fluorescent protein (both inserted at the F14.5L locus), beta-galactosidase (inserted at the J2R locus, which encodes thymidine kinase), and beta-glucuronidase (at the A56R locus, which encodes hemagglutinin) not only attenuate the virus but create multiple possibilities for detection of the viral particle in cells. GLV-1h68 successfully replicated in and eradicated solid tumors when applied in nude mice (Zhang et al. 2007, Yu YA et al. 2009, Yu Z et al. 2009) and was therefore chosen to be evaluated for human application in clinical trials.

3.4 Utilizing microRNAs in oncolytic virotherapy

Oncolytic viruses may face issues when administered to human patients such as innate inflammatory reactions to viral particles or the body´s B- and T-cell responses to viral antigens presented by the cells they are replicating in which may create the need to increase the viral load or combine the therapy with immunosuppressive drugs. The application of high viral doses can be detrimental to the health of patients as demonstrated in various clinical trials using adenoviruses (Harvey et al. 2002, Yen et al. 2000, Raper et al. 2002, Mickelsen 2002). Although in recent years the safety of systemically administered oncolytic viruses has increased (Kumar et al. 2008, Markert et al. 2000, Park et al. 2008, Vitell-Pedersen et al. 2011) new approaches to further limit viral replication to cancer cells may aid the significance of oncolytic virotherapy in the fight against cancer. This goal was achieved by expressing microRNA target structures in virus genomes, thereby inhibiting replication in tissues expressing cognate microRNAs (Edge et al. 2008, Kelly et al. 2008 and 2010, Lee et

al. 2009, Ylösmaki et al. 2008). Leber et al. (2011) could successfully control oncolytic measles virus replication by inserting microRNA-7 target sequences shown to be down-regulated in glioblastoma multiforme. Furthermore, microRNAs expressed by viral vectors could be used as biomarkers in cancer profiling. This was successfully conducted by Rosenfeld et al. (2008) who used miRNA expression levels in multiple primary and metastatic cancers to construct a miRNA classifier which identified cancer metastases by their site of origin, a potential utility for individuals with cancer of unknown primary origin. Another potential use of microRNAs in viral vector targeting of cancer could be the delivery of microRNAs known to be down-regulated or abundant in cancers, thereby altering cancer cell behavior which has been shown for adenoviruses (Roth et al. 2006, Kota et al. 2009) and influenza virus (Fulmer et al. 2010). In the case of oncolytic vaccinia viruses which replicate exclusively in the cytoplasm the nuclear splicing machinery cannot be utilized to produce mature microRNAs. In this case, the incorporation of self-cleaving enzymes such as ribozymes which could replace the nuclear splicing machinery might be necessary to yield functional mature microRNAs without the need to incorporate nuclear polymerases (Suryawanshi et al. 2010). The generation of a microRNA sensitive vaccinia virus could be the first step to achieving the goal of assembling components of the nuclear splicing machinery in the cytoplasm of eukaryotic cells by successful expression and processing of a target mRNA synthesized by vaccinia virus which is recognized by endogenous miRNAs.

3.5 Aim of the study: The generation of a recombinant vaccinia virus carrying complementary repeats of human miRNA-let7a with an increased tropism towards replication in cancer cells

The diversity of cancer calls for innovative approaches to target cancer cells specifically in addition to traditional therapies such as chemotherapy or radiation therapy which commonly cause strong side effects in treated patients. Treatment of various types of cancer using oncolytic viruses represents a promising way to target cancerous cells and lyse them without destroying healthy tissue. The recombinant oncolytic vaccinia virus GLV-1h68 displayed strong oncolytic potential and the ability of easy detection within the body via gfp expression and is therefore currently in evaluation for clinical application in humans with solid tumors. However, toxicity is still an issue, especially when virotherapy is combined with traditional therapies known to be associated with low drug tolerance. Several types of cancer display a unique microRNA expression pattern and a significant up- or downregulation of endogenously expressed microRNAs. The behavior of cancer cells could be manipulated by either adding microRNAs known to inhibit cancer cell spread and proliferation or by suppressing cancer promoting microRNAs (Kota et al. 2009, Ma et al. 2007). Edge et al. 2009 successfully demonstrated that an oncolytic vesicular stomatitis virus expressing microRNA-let7a complementary repeats displayed a tumor specific replication (fig. 6). It should be investigated whether or not further cancer cell specificity of a recombinant vaccinia virus strain could be obtained without compromising its oncolytic activity using endogenous microRNA interference. To achieve this goal a quadruple complementary sequence repeat of mature human microRNA let-7a would be cloned in front of the stop codon in the 3'end of the

vaccinia virus D4R gene, using a GLV-1h68 derivative, GLV-1h190, as parental strain yielding the new recombinant virus GLV-1h250. The D4R gene belongs to the group of early transcribed vaccinia genes and encodes for an essential enzyme, uracil DNA glycosylase, which catalyzes the removal of uracil residues from double-stranded DNA, preventing a virus defective in D4R from entering into the intermediate and late phase of replication under non complementing conditions and is considered essential for viral replication (Ellison et al. 1996, Millns et al. 1994).

In this study, a GLV-1h68 derivative, GLV-1h190 would be used as backbone to create a new virus, GLV-1h250, which would carry a quadruple human miRNA-let-7a complementary sequence repeat cassette. With help of the cell´s own microRNA machinery GLV-1h250 should be forced to replicate exclusively in cancerous tissue. This virus would display strong oncolytic activity in cancer cells while sparing healthy cells. Its replication would be strongly decreased in relation to GLV-1h68 and GLV-1h190 when incubated with Endometrial Regenerative Cells (ERC) *in vitro*. These cells are considered healthy with a high let-7a expression. Endogenous let-7a should recognize its target structures on the viral mRNA, thereby degrading the mRNA leading to a significant decrease in viral titers after uptake and replication. This phenomenon should be dose-independent. In contrast, when GLV-1h250 replicated in A549 lung adenocarcinoma cells the virus should display a strong oncolytic potential comparable to its parental strain GLV-1h190 and GLV-1h68 to further emphasize a let-7a-dependent replication of the virus. In an *in vivo* setting with A549 lung adenocarcinoma bearing nude mice, retro orbitally administered GLV-1h250 should replicate solely in tumorous tissue which would be detectable via fluorescent protein expression and viral titration of *ex vivo* samples. No viral particles should be

found outside the tumorous tissue suggesting a microRNA-let7a-dependent replication of the virus. This observation should be possible even when high amounts of virus are used. The animals would show no side effects characterized by no signs of pox formation, a steady body weight through out the entire study and a good overall health status.

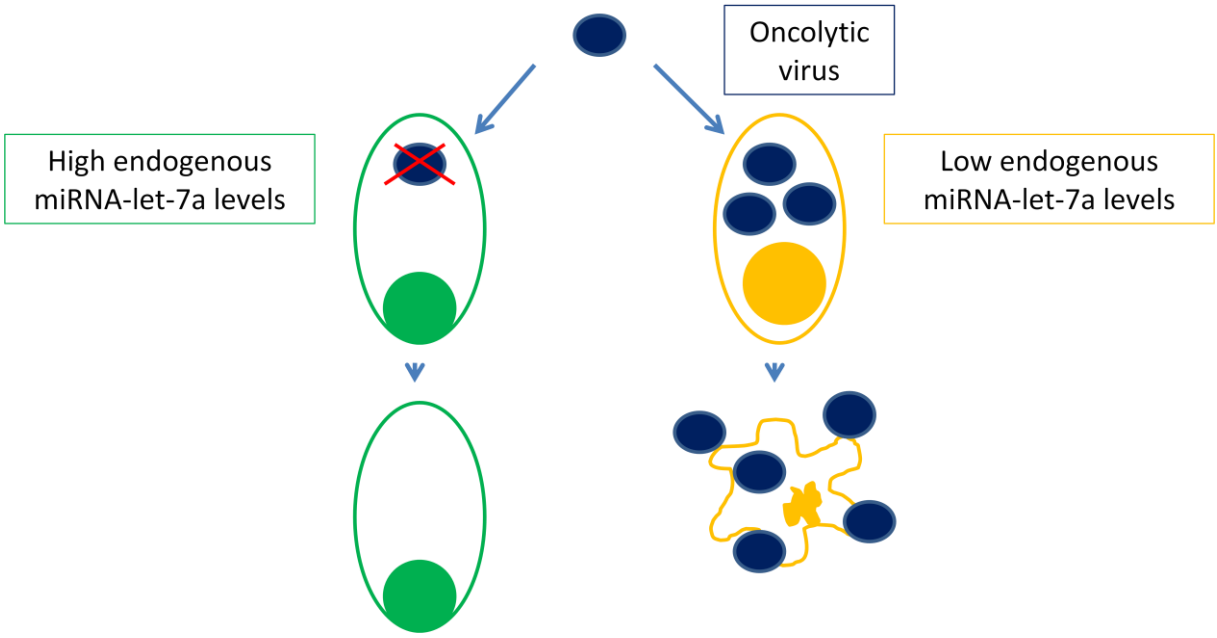


Figure 6: Mode of action of GLV-1h250

Viral particles in a healthy cell cannot replicate due to post translational repression mediated by high endogenous let-7a levels. In cancer cells the replication of the virus is not repressed due to low endogenous miRNA-let-7a levels, therefore the replication is increased.

Oncolytic viruses carrying inserted microRNA targets in their genomes could be controlled in their replication behavior to preferentially target cancer cells. This was applied to further increase the safety of the next generation recombinant oncolytic vaccinia virus GLV-1h250 which should display solid oncolytic potential with reduced toxicity showing strongly reduced replication potential in healthy cells and tissues.

This should be demonstrated *in vitro* and *in vivo* in comparison to its parental strain GLV-1h190 and GLV-1h68, the vaccinia virus used in clinical trials.

4 Materials

4.1 Chemicals and enzymes

Materials	Manufacturer
100bp DNA Ladder	New England Biolabs
1kb DNA Ladder	New England Biolabs
2-log DNA Ladder	New England Biolabs
2-Mercaptoethanol	Fisher Scientific
5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal)	Stratagene
5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt (X-GLCA)	RPI Research Products
15% TBE Urea gels	Invitrogen
Accuprime™ Pfx SuperMix	Invitrogen
Acetic Acid	VWR
Ampicillin	Sigma
Antibiotic-Antimycotic Solution	Cellgro
Biorad Protein Assay Standard I	Biorad
Bromophenol Blue	Aldrich Chemical Company
Carboxymethyl Cellulose Sodium Salt	MP Biomedicals
Chloroform	Fisher Scientific
Citric Acid	Sigma
Clearslip Mounting Media	IMEB Inc.

Coomassie Brilliant Blue R250	Sigma
Crystal Violet	Sigma
Difco™ Agar	BD
Difco™ LB Broth, Miller	BD
Difco™ Skim Milk	BD
DIG UltraHyb Hybridization buffer	Ambion
DMEM medium 1x	Cellgro
Dulbecco's Phosphate Buffered Saline (DPBS) 1x	Cellgro
EDTA	Fisher Scientific
Eosin-Y	Richard-Allan Scientific
Ethidium Bromide	Sigma
Fetal bovine serum	Omega Scientific
Formaldehyde (37%)	EMD
Formalin 1:10 Dilution, Buffered	Fisher Diagnostics
Formamide	EMD
FuGENE® Transfection Reagent	Roche
Glucose	Cellgro
Glycerol	Fisher Scientific
Hematoxylin QS	Vector
HEPES	Gibco
HyClone® HyPure™ Cell Culture Grade Water	Thermo Scientific
Hydrochloric Acid Solution 2N	VWR
Hydrogen Peroxide Solution	Sigma
Hydroxymethylaminomethanehydrochloride (Tris-HCl)	Fisher Scientific

Hypoxanthine	Sigma
Kanamycin	Sigma
Lipofectamine 2000	Invitrogen
Maleic Acid	Fisher Scientific
Magnesiumchloride	J.T.Baker
Methanol, Absolute	Sigma Diagnostics
Modified Mayer's Hematoxylin	Richard-Allan Scientific
Mycophenolic Acid	Sigma
Native PAGE™ 20x Cathode Buffer Additive	Invitrogen
Native PAGE™ 20x Running Buffer	Invitrogen
Native PAGE™ Sample Additive	Invitrogen
Native PAGE™ Sample Buffer	Invitrogen
NorthernMax™ High Stringency Buffer	Ambion
NorthernMax™ Low Stringency Buffer	Ambion
Novex TBE Running Buffer	Invitrogen
Novex TBE Urea Sample Buffer	Invitrogen
NuPAGE® 20x Transfer Buffer	Invitrogen
NuPAGE® MOPS SDS 20x Running Buffer	Invitrogen
OmniPur Ethyl Alcohol Pure	EMD
OmniPur® Agarose	EMD
OPTI-MEM® Medium	Gibco
Paraplast Tissue Embedding Medium	McCormick Scientific
Paraformaldehyde	Sigma
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	Sigma
Polyplus Transfection Reagent	Polyplus

Precision Plus Protein™ Standard	Biorad
Proteinase Inhibitor Cocktail Tablets	Roche
Proteinase k	Sigma
Quick T4 DNA Ligase	New England Biolabs
Restriction Enzymes	New England Biolabs
RPMI Medium 1640 1x	Cellgro
RNAse Away	MBP
S.O.C. Medium	Invitrogen
Sodium Acetate	Fisher Scientific
Sodium Carbonate	Fisher Scientific
Sodium Chloride	Fisher Scientific
Sodium Citrate	Fisher Scientific
Sodium Dodecylsulfate (SDS)	Fisher Scientific
Sodium Hydrogencarbonate	Fisher Scientific
Sodium Hydroxide	Fisher Scientific
Sodium Pyruvate	Cellgro
Sucrose	Sigma
Taqman MicroRNA Assay microRNA181a	ABI custom
Taqman MicroRNA Assay microRNA 335	ABI custom
Taqman MicroRNA Assay microRNA U6	ABI
Taqman MicroRNA Assay microRNA let-7a	ABI
Taqman Universal Master Mix, no UNG	ABI
TBE Buffer 10x	Sigma
TMB	Sigma
Trichloroacetic Acid	VWR

Tris (Base)	Fisher Scientific
Tris-Borate-EDTA (TBE) Buffer	Sigma
Triton-X 100	Sigma
TRIzol® Reagent	Invitrogen
Tween-20	Biorad
Xanthine	Calbiochem
Xylazine 20 Injection	Butler
Xylene Substitute	Sigma
Z-Competent™ *E. coli* DH5α Cells	Zymo Research

4.2 Cell lines and cell culture media

A549 cells:	human lung carcinoma (adherent)
CV-1-cells:	green monkey kidney fibroblasts (adherent)
ERC:	human endometrial regenerative cells (adherent)
HEK293:	human embryonic kidney epithelial cells (adherent)

Cell culture media:

A549:	500ml RPMI-1640
	10% FBS
	1% Antibiotics-Antimycotics

CV-1:	500ml DMEM
	10% FBS
	1% Antibiotics-Antimycotics
ERC:	500ml DMEM/F12
	10% FBS
	2 mM L-Glutamine
	10 mM HEPES
	1% Antibiotics-Antimycotics
HEK 293:	500ml EMEM
	10% FBS
	1% Sodium Pyruvate
	Non-essential Amino Acids
	1% Antibiotics-Antimycotics

4.3 Buffers and solutions

1x SDS Buffer:	83.3 ml H_2O
	1.0 ml1 M Tris-HCl
	4.0 ml 5 M NaCl
	0.2 ml 0.5 M EDTA
	10.0 ml 10% SDS
	add 15 µl of 10 mg/ml proteinase K and 0.35 µl

β-Mercaptoethanol per ml fresh prepared solution.

2x Triton X-100 Buffer: 90.52 ml H_2O

8.0 ml 0.5 M EDTA

1.0 ml Triton X-100

add 4.8 µl of β-mercaptoethanol per ml fresh prepared solution.

3.7% Paraformaldehyde: 18.5 g Paraformaldehyde

500 ml PBS

filter through 0.45 micron filter

β-galactosidase staining solution: 1.5 ml Solution B

0.5 ml PBS

30 µl X-Gal

Coomassie Destaining Solution: 40% Methanol

7% Acetic Acid

Coomassie Staining Solution: Coomassie Brilliant Blue R-250 0.025%

Methanol 40%

Acetic Acid 7%

Crystal Violet Solution:	1.3 g Crystal violet
	50 ml Ethanol
	stir at room temperature for 1 hour
	300 ml 37% Formaldehyde
	ad 1L with Aqua dest.
	stir at room temperature overnight
Hypoxanthine (670x):	50 mg Hypoxanthine
	5 ml NaOH 0.1 N
	filter sterilize and store at -20˚C
MPA (400x):	50 mg Mycophenolic Acid
	5 ml NaOH 0.1 N
	filter sterilize and store at -20˚C
PBST:	0.05% Tween-20 in PBS
Protein gel Stripping Buffer:	100 mM 2-Mercaptoethanol
	2% SDS
	62.5 mM Tris-HCl
	Adjust to pH 6.7
RIPA Buffer:	150 mM NaCl
	10 mM Tris, pH 7.2
	0.1% SDS

1.0% Triton X-100

1.0% Deoxycholate

5 mM EDTA

Add one tablet of Proteinase Inhibitors (Roche)

to 2 ml PBS and dissolve

add 4 µl per ml of RIPA buffer

SDS-Loading Buffer: 5 ml1 M Tris-HCl pH 6.8

10 ml Glycerin 20%

20 ml SDS 4%

0.001 g Bromphenole Blue

add 50 ml distilled H_2O

add 200 mM DTT

Solution B: 2 mM $MgCl_2$

5 mM $K_3Fe(CN)_6$

5 mM $C_6FeK_4N_6$ x 3 H_2O

Viral Plaque Overlay Medium

For CV-1 Cells: 15 g Carboxy-Methyl Cellulose

weighed in 1 L bottle and autoclaved

1000 ml DMEM

Antibiotic-Antimycotic solution 10 ml

stir at room temperature until dissolved

50 ml FBS

stir at room temperature for 2-4 hours

Xanthine (40x): 500 mg Xanthine

50 ml NaOH 0.1 N

filter sterilize and store at -20˚C

4.4 Kits

Kit	Manufacturer
Biorad D_C Protein Assay	Biorad
DIG Luminescent Detection Kit	Roche
DNA Clean & Concentrator™-5Kit	Zymo Research
DNA-free™ Kit	Ambion
MicroRNeasy Mini Kit	Qiagen
Native PAGE® Sample Prep Kit	Invitrogen
Opti-4CN™ Substrate Kit	Biorad
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen
RNase-free DNase Set	Qiagen
RNeasy® Mini Kit	Qiagen
SuperScript™ First-Strand Synthesis System for RT-PCR	Invitrogen
Zero Blunt® TOPO® PCR Cloning Kit	Invitrogen
Zymoclean™ Gel DNA Recovery Kit	Zymo Research

4.5 Synthetic Oligonucleotides

D4R for: 5'-ATG GAT CCA TGA ATT CAG TGA CTG TAT CAC ACG-3'

D5R rev: 5'-ACT AGT CCT ATA TAC GGC AGT GTC TAT CGA TC-3'

Let-7a for: 5'- AAC TAT ACA ACC TAC TAC CTC ACG ATA ACT ATA CAA CCT ACT ACC T CA ACC GGT AAC TAT ACA ACC T-3'

Let-7a rev: 5'-TGA GGT AGT AGG TTG TAT AGT TGT GAT GAG GTA GTA GGT TGT ATA GTT ACC GGT TGA GGT AGT AGG-3'

D5R/let-7a for: 5'-ACT ACC TCA TCA CAA CTA TAC AAC CTA CTA CCT CAA GAT CCT GCT TTA GTG AAA TTT TAA CTT GTG T-3'

D4R/let-7a rev: 5'- TTG TAT AGT TAT CGT GAG GTA GTA GGT TGT ATA GTT TTA CTT GTC GTC TAG GTC CTT GTA GTC-3'

D4R qPCR for: 5' TTC TCG AAT ATA CGG GCC AAG T-3'

D4R qPCR rev: 5'-CCG CTG GAT GAT ATC CGA CTA-3'

F4L qPCR for: 5'-CCG GAT CAT TCG CTT CCA TA-3'

F4L qPCR rev: 5'-AAA CGT GAG TCC GGG CAT TA-3'

A21L qPCR for: 5'- AAA CAG TCT ATC ATC GAC ACA AAT GAA-3'

A21L qPCR rev: 5'-CGA ACG AGC CGC ATA CG-3'

All PCR primers were synthesized by IDT, all qPCR primers were synthesized by Applied Biosystems.

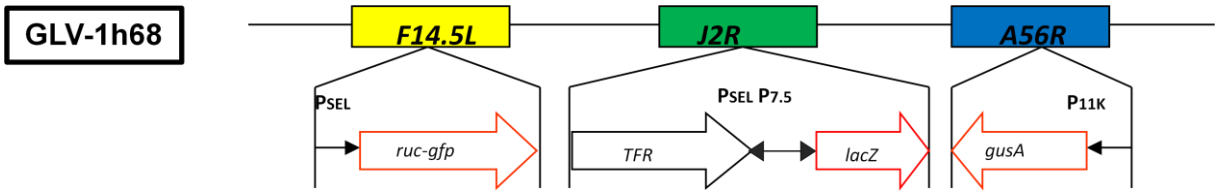
4.6 Antibodies for Western blot and DIG-labeled qPCR probes

Antibody	Source	Manufacturer
anti-vaccinia D4R	rabbit	Genescript custom
anti-β-actin	mouse	Sigma
anti-mouse IgG peroxidase conjugate	goat	Sigma
anti-rabbit conjugated to HRP	goat	Biorad
Probe	**Label**	**Manufacturer**
Taqman vacv-d4r	Digoxigenin	ABI custom
Taqman vacv-a21l	Digoxigenn	ABI custom
Taqman vacv-f4l	Digoxigenin	ABI custom

4.7 Recombinant viral constructs

GLV-1h68 is a recombinant oncolytic vaccinia virus generated by Genelux Corporation, San Diego, CA. Three expression cassettes encoding marker genes were inserted into the *F14.5L*, *J2R*, and *A56R* loci of the viral genome. The *F14.5L* locus contains a *Renilla* luciferase *Aequorea* green fluorescent protein fusion gene (*RUC-GFP*) controlled by a synthetic early/late promoter. The ß-galactosidase gene (*lacZ*) was inserted into the J2R locus under the control of the $P_{7.5}$ promoter and the ß-glucuronidase gene (*gusA*) is located in the A56R locus controlled by the P_{11K} promoter. The transferrin receptor (TFR) cDNA was inserted in reverse orientation to

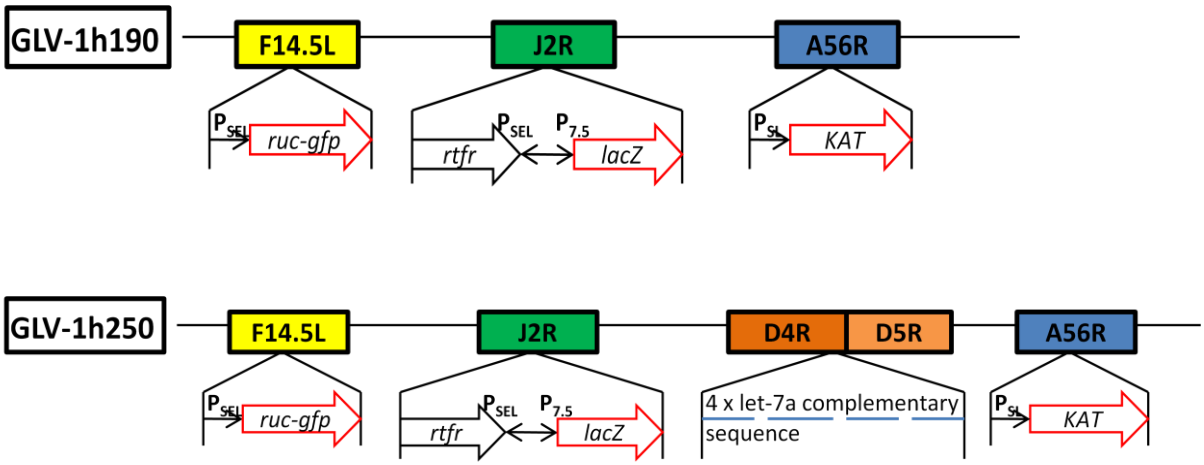
the vaccinia synthetic early/late promoter to serve as a negative control for a TFR-expressing recombinant virus (Zhang *et al*., 2007).



The vaccinia virus strain GLV-1h190 was derived from GLV-1h68 as a parental virus but instead of the β-glucuronidase gene in the *A56R* locus the *katushka* gene (*KAT*) encoding for the red fluorescent Turbo FP 635 protein (ex/em max 588/ 635 nm) under the control of the synthetic late promoter was inserted.

The vaccinia virus strain GLV-1h190 was then used as a parental strain for the generation of GLV-1h250. In this vaccinia virus strain none of the inserted marker genes were removed or replaced. Instead, this virus contains an insertion of 4 x mature hsa-miR-let-7a complementary repeats after the stop codon in the 3'-UTR of the *D4R* gene. When recognized and bound by the host cell miR-let-7a a degradation of the viral mRNA and subsequently an inhibition of the replication of the virus should take place (Edge et *al*. 2008).

Shown below is the location of the quadruple let-7a complementary sequence insertion relative to the loci used for marker gene insertion within GLV-1h250 in comparison with its parental strain GLV-1h190. The generation of the virus GLV-1h250 is described in the Methods part.



4.8 Bacterial strains

E. coli Omnimax pTolC

E. coli Omnimax pTolC-EC20

E. coli Top10

4.9 Laboratory animals

Male athymic nude FoxN1 mice were used for the *in vivo* studies. This mouse strain is characterized by an autosomal recessive mutation in the *nu* locus on chromosome 11 leading to a completely hairless phenotype. Also, these animals feature a rudimental and dysfunctional thymus which expresses itself as a T-cell deficiency in contrast to B-cell function, which is normal in athymic nude FoxN1 mice. Due to the defects in the immune system of the mouse, athymic nude FoxN1 mice are suited as

adequate laboratory animals in oncology, immunology and other fields of biomedical research. Another advantage of the used mouse model is that xenotransplants will not be rejected by the mouse.

Phenotype of an athymic nude FoxN1 mouse (http://www.harlan.com)

All animals were purchased from Harlan. Mice were held and maintained in accordance with animal welfare regulations under the approved protocol by the Institutional Animal Care and Use Committee of Explora Biolabs (San Diego Science Center).

4.10 Laboratory equipment and other materials

Equipment	Manufacturer
Balance PL1501-S	Mettler Toledo
Bio Doc-It™ System	UVP
Biosafety Cabinet	The Baker Company Inc.
Cell Culture Cluster 24-well Costar 3526	Corning Inc.
Cell Culture Cluster 6-well Costar 3516	Corning Inc.
Cell Culture Cluster 96-well Costar 3595	Corning Inc.

Cell Culture Flask 75cm^2	Corning Inc.
Cell Culture Flask 225cm^2	Corning Inc
Cell Scraper	Corning Inc.
Cell Spreader	VWR International
Centrifuge Sorvall RC 6 Plus	Thermo
Centrifuge Centra CL2	Thermo
Centrifuge Micro CL 21	Thermo
Combitips Plus 25ml	Eppendorf
Cryotubes 2ml	Nalgene
Digital Caliper	VWR
Digital Dry Bath Incubator	Boekel Scientific
Dish 10cm	Fisher Scientific
Falcon 15ml Tubes	BD
Falcon 50ml Tubes	BD
Fluorescence Microscope IX71	Olympus
Heater	VWR International
Hotplate Stirrer 375	VWR Scientific Products
Illumatool Tunable Lighting System	Lightools Research
Incubator HERA Cell 150	Thermo Electron Corporation
Incubator Shaker C25	New Brunswick Scientific
Insulin SyringeU-100 29G1/2	BD
MagNA Lyser	Roche
MagNA Lyser Green Beads	Roche
MicroAmp Fast Opitical 96-well reaction plate	ABI
Microfuge Tubes 2.0ml	Avant

Microfuge Tubes Easy Open Cap 1.5ml	Sarstedt
Microplate Reader SpectraMax MS	Molecular Devices
Microwave Carousel	Sharp
Multipipette	Eppendorf
Optical Adhesive Covers	ABI
Parafilm Laboratory Film	Pechiney Plastic Packaging
pH Meter Accumet AR15	Fisher Scientific
Photometer Biomate3	Thermo Spectronic
Pipet Aid	Drummond
Pipettes 1-20µl, 20-200µl, 100-1000µl	Gilson
Pipet Tips 200-1000µl, 100µl, 10µl	VWR International
Pipettes 25ml, 10ml, 5ml	Corning Inc.
Power Pac 200	Biorad
Precsat Novex® 10% TBE-Urea Gel	Invitrogen
Precast NuPAGE® 12% Bis-Tris Gel	Invitrogen
PVDF Membrane Filter Paper Sandwich 0.2µm	Invitrogen
Red Roller II Hybridization oven	Hoefer
Rocking Platform	VWR International
Sonifier 450	Branson
StepOnePlus Real-time PCR system	ABI
Sterile Disposable Scalpel	Sklar Instruments
Syringe 1ml	BD
Syringe 5ml	BD
Syringe Driven Filter Unit Millex®-VV PVDF 0.1µm	Millipore
Thermocycler Mastercycler Personal	Eppendorf

Tissue Culture Dish 60mm	BD
Tissue Embedding Center	Reichert – Jung
Tissue Grinder	Kimble
Tissue Processing/Embedding Cassettes w. Lid	Simport
UV-Stratalinker 1800	Stratagene
Vortex VX100	Labnet
Water Bath	Boekel Scientific
Water Bath Isotemp	Fisher Scientific
X Cell Sure Lock™	Invitrogen

5 Methods

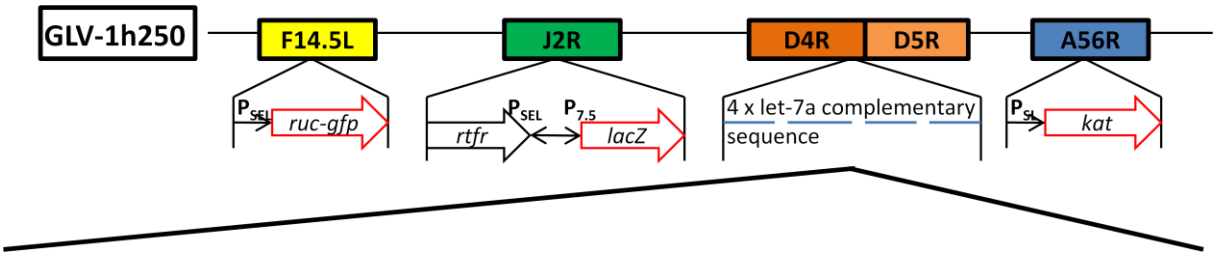
5.1 Generation of recombinant vaccinia virus GLV-1h250

A virus with new and different properties compared to its parental strain was created to increase the performance and safety of viruses already in use. Therefore a transfer vector was designed to deliver the desired changes in sequence into a cell and there recombine with a virus strain chosen to be the parental backbone to create a virus carrying and expressing the introduced genes.

5.1.1 Generation of the Transfer Vector

Cloning strategy:

Shown below is a map with the loci used for the expression of marker genes and the let-7a complementary sequence insertion site. The D4R gene is highlighted blue and contains a Flag-Tag (lower case letters) for additional detection possibilities at its 3´end followed by the let-7a complementary repeat sequence cassette highlighted purple. The D5R gene is highlighted green. Sites of restriction enzymes are highlighted red and the 5´end of each primer is underlined.



ggatcc

ATGAATTCAGTGACTGTATCACACGCGCCATATACTATTACTTATCACGATGATTGGGAACC

AGTAATGAGTCAATTGGTAGAGTTTTATAACGAAGTAGCCAGTTGGCTGCTACGAGACGAGA

CGTCGCCTATTCCTGATAAGTTCTTTATACAGTTGAAACAACCGCTTAGAAATAAACGAGTA

TGTGTGTGTGGTATAGATCCGTATCCGAAAGATGGAACTGGTGTACCGTTCGAATCACCAAA

TTTTACAAAAAAATCAATTAAGGAGATAGCTTCATCTATATCTAGATTAACCGGAGTAATTG

ATTATAAAGGTTATAACCTTAATATAATAGACGGGGTTATACCCTGGAATTATTACTTAAGT

TGTAAATTAGGAGAAACAAAAAGTCACGCGATTTACTGGGATAAGATTTCCAAGTTACTGCT

GCAGCATATAACTAAACACGTTAGTGTTCTTTATTGTTTGGGTAAAACAGATTTCTCGAATA

TACGGGCCAAGTTAGAATCCCCGGTAACTACCATAGTCGGATATCATCCAGCGGCTAGAGAC

CGCCAATTCGAGAAAGATAGATCATTTGAAATTATCAACGTTTTACTGGAATTAGACAACAA

GGCACCTATAAATTGGGCTCAAGGGTTTATTTATgactacaaggacctagacgacaagTAAA

ACTATACAACCTACTACCTCAcgatAACTATACAACCTACTACCTCAaccggtAACTATACA

ACCTACTACCTCAtcacAACTATACAACCTACTACCTCAagatccTGCTTTAGTGAAATTTT

AACTTGTGTTCTAAATGGATGCGGCTATTAGAGGTAATGATGTTATCTTTGTTCTTAAGACT

ATAGGTGTCCCGTCAGCGTGCAGACAAAATGAAGATCCAAGATTTGTAGAAGCATTTAAATG

CGACGAGTTAGAAAGATATATTGAGAATAATCCAGAATGTACACTATTCGAAAGTCTTAGGG

ATGAGGAAGCATACTCTATAGTCAGAATTTTCATGGATGTAGATTTAGACGCGTGTCTAGAC

GAAATAGATTATTTAACGGCCATTCAAGATTTTATTATCGAGGTGTCAAACTGTGTAGCTAG

ATTCGCGTTTACAGAATGCGGCGCCATTCATGAAAATGTAATAAAATCCATGAGATCTAATT

TTTCATTGACTAAGTCTACAAATAGAGATAAAACAAGTTTTCATATTATCTTTTTAGATACG

TATACCACTATGGATACATTGATAGCTATGAAACGAACACTATTAGAATTAAGTAGATCATC

TGAAAATCCACTAACCAGATCGATAGACACTGCCGTATATAGGactagtAACGGCCGCCAGT

GTGCTGGAATTCGGCTT

Restriction sites:

ggatcc: BamHI

agatcc: AgeI

actagt: SpeI

Primers:

D4R for:

5'-ATggatccATGAATTCAGTGACTGTATCACACG-3'

Let-7a for:

5´-AACTATACAACCTACTACCTCACGATAACTATACAACCTACTACCTCAACCGG

TAACTAT ACAACCT-3'

D4R/let-7a rev:

5-TTGTATAGTTATCGTGAGGTAGTAGGTTGTATAGTTTTACTTGTCGTCTAGGTC

CTTGTAG TC-3'

D5R/let-7a for:

5’-ACTACCTCATCACAACTATACAACCTACTACCTCAagatccTGCTTTAGTGAAATT TTAAC TTGTGT-3’

Let-7a rev:

5’-TGAGGTAGTAGGTTGTATAGTTGTGATGAGGTAGTAGGTTGTATAGTTACCGG TTGAGG TAGTAGG-3’

D5R rev:

5’-ACTAGTCCTATATACGGCAGTGTCTATCGATC-3’

The transfer vector pCR2.1-D3R-D4R-Flag-D5R-gpt7 used as a backbone in this study was designed and generated by Genelux Corporation, San Diego, CA. This vector contained the sequences for the Vaccinia virus genes *D3R*, *D4R* and *D5R*. Before the stop codon at the 3’end of the D4R gene the vector carries a Flag tag for detection purposes. Following the *D5R* gene the vector carried the sequence for the *E.coli gpt* gene under control of the Vaccinia Virus p7.5 early promoter for screening purposes.

The let-7a complementary sequence expression cassette would be inserted behind the stop codon at the 3’end of the *D4R* gene to generate a recombinant virus showing a replication deficiency in healthy cells which display high expression levels of microRNA let-7a. The unique restriction sites BamHI at the 3’ end of the D4R gene and SpeI at the 5’ end of the D5R gene were already present on the transfer vector. The restriction sites allowed cutting of the DNA at the exact position and

made it possible to ligate the sequence with any vector carrying the same restriction sites.

Due to the length and the rather complicated secondary structure of the sequence, 4 long partially overlapping primers were designed and amplified by PCR using the following setup and programs:

Template	1µl pCR2.1-D3R-D4R-Flag-D5R-gpt7	1µl pCR2.1-D3R-D4R-Flag-D5R-gpt7
Primer 1	0.5 µl Let-7a for	0.5 µl D5R-let-7a for
Primer 2	0.5 µl D4R-let-7a rev	0.5 µl Let-7a rev
Accuprime pfx Supermix	22.5 µl	22.5 µl
PCR program	5‘ 95°C initializing 35x 30‘‘ 94°C denaturing 30‘‘ 55°C annealing 1‘ 68°C elongation 5’ 68°C ∞ 4°C	5‘ 95°C initializing 35x 30‘‘ 94°C denaturing 30‘‘ 55°C annealing 1‘ 68°C elongation 5’ 68°C ∞ 4°C

The products of both PCR reactions were combined and used as a template for the next PCR:

Template	1 µl of each generated PCR product
Primer 1	0.5 µl D4R for
Primer 2	0.5 µl D5R rev
Accuprime pfx Supermix	22.5 µl
PCR program	5‘ 95°C initializing 35x 30‘‘ 94°C denaturing 30‘‘ 52°C annealing 2‘ 68°C elongation 5’ 68°C ∞ 4°C

After the second round of PCR, the PCR product was purified using the Gel Recovery Kit (ZymoResearch). Four µl of the blunt end PCR product was mixed together with 1 µl pCRII®-Blunt-TOPO vector and 1 µl Salt Solution in the TOPO® Cloning reaction by incubating for five minutes at room temperature. Two microliters of the TOPO® Cloning reaction were added to one vial of Top10-One Shot Chemically Competent *E.coli* (Invitrogen). The reaction was incubated on ice for five minutes. The cells were heat-shocked for 30 seconds at 42 ˚C and then transferred back on ice. Subsequently 250 microliters of S.O.C. medium were added and the tube was shaken horizontally (200 rpm) at 37 ˚C for one hour. Fifty microliters of the

transformation were spread out on a pre-warmed LB-plate containing 50 µg/ml kanamycin. Plates were incubated over night at 37 ˚C. Several colonies were picked for overnight bacterial cultures in LB medium with 50 µg/ml kanamycin and incubated in a rotating incubator at 37 ˚C and 200 rpm. The overnight cultures were used to isolate the DNA with the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) according the manufacturer's instructions. The isolated DNA was digested with the restriction enzyme EcoRI and the clones with inserts at the expected sizes were sent out for sequencing.

One of the positive clones with confirmed sequence, D4R-let7acomp-D5R, was used for subcloning into the D4R transfer vector pCR2.1-D3R-D4R-D5R-gpt7 for the subsequent homologous recombination with the parental strain GLV-1h190 in CV-1 cells. Therefore both, the DNA of the insert and the vector, were digested with the enzymes BamHI and SpeI. Since both enzymes needed different buffer compositions a sequential digest was performed. The insert DNA and the vector DNA were digested with BamHI in NEB buffer 3 over night at 37°C and subsequently purified using the DNA Clean and Concentrator™-Kit (Zymo Research). After purification, the insert and the vector were digested with SpeI in NEB buffer 4 at 37°C over night. The vector DNA was treated with 1 µl CIP in NEB buffer 3 over night at 37°C. The CIP reaction helps to avoid ligation of the vector with itself due to catalyzation of the removal of 5´ phosphate groups from DNA, RNA, ribo- and deoxyribonucleoside triphosphates by the Alkaline Phosphatase (Sambrook et al. 1989). The digested insert and vector DNA was separated by gel electrophoresis. Bands of expected were cut out and purified with the Zymoclean™ *Gel* DNA Recovery Kit (Zymo Research) and used for ligation.

Ligation was performed with the Quick T4 DNA Ligase (New England Biolabs) according to the manufacturer's instructions. After the ligation the newly created plasmid DNA was transformed into Top10-One Shot Chemically Competent *E. coli* (see above) using 50 µg/ml kanamycin containing LB-plates and grown over night at 37°C. The following day several colonies were picked and grown over night shaking at 37°C and 200 rpm. After isolation of the DNA (see above) a control restriction was performed using AgeI enzyme, which displayed one restriction site within the let-7a complementary sequence and one outside within the *D5R* gene. After verifying the cut DNA sizes, the DNA of the colonies showing the right sized insertion were sent out for sequencing. The sequence was verified and the successfully generated plasmid DNA was used for homologous recombination with GLV-1h190 to create a new virus.

5.1.2 Homologous recombination of plasmid DNA with parental virus GLV-1h190

For the homologous recombination with the parental virus GLV-1h190, CV-1 cells were seeded the day before in 6-well plates at a concentration of 4.0 x 10^5 cells/well. First the virus was diluted to a concentration of 1x10^5 pfu/ml and the cells were incubated with 0.5 ml of virus containing media. The plates were incubated for one hour at 37 ˚C and 5% CO_2 and shaken every 20 minutes for better distribution of the virus.

Plasmid DNA was co-transfected 30 minutes after the addition of the virus. The DNA was prepared by pipetting 12 µl of FuGENE transfection reagent (Roche) to 88 µl of OPTI-MEM media. After vortexing and an incubation at room temperature for five minutes 2 µg of DNA were added. Then the mixture was vortexed again and incubated at room temperature for 20 minutes. After one hour of incubation the cells were washed twice with OPTI-MEM medium and the 1.5 ml of OPTI-MEM was added to the cells. The DNA mix was added and the plates were incubated at 37 ˚C and 5% CO_2 for one to two days until all the cells showed virus replication. Then the cells were harvested and frozen at -80 ˚C.

5.1.3 Plaque selection

The transfected cell lysate was frozen and thawed three times to ensure cell break up. Then it was sonified 3 times for 1 minute each using 100% power with 30 seconds break in between each sonification step to further break up the cells and break up virus aggregates. CV-1 cells seeded in 6-well-plates were treated with the three drugs mycophenolic acid (MPA), xanthine, and hypoxanthine the day before the addition of the virus strain. MPA is an inhibitor of purine metabolism and blocks the pathway for GMP synthesis. Therefore it interferes with the replication of vaccinia and reduces the size of the virus plaques. However, this effect could be overcome by expression of the *E.coli gpt* gene in the presence of xanthine and hypoxanthine. The *gpt* gene was inserted next to the gene of interest and outside the vaccinia DNA flanking regions. Under these conditions only the single cross-over recombinant virus would express xanthine-guanine phosphoribosyltransferase (gpt), which would lead

to enrichment over the parental virus (Falkner and Moss, 1990). One day after pretreatment with these drugs, the cells were incubated with 50, 10 and 2 µl of virus containing cell lysate. After one hour of incubation at 37 ˚C and 5% CO_2 2 ml of overlay medium were added and the cells were incubated for five days at 37 ˚C and 5% CO_2. Six single plaques were then picked by aspirating 150 µl of medium surrounding single plaques and adding them into tubes with 500 µl of DMEM medium. These samples were then frozen and thawed three times and after sonication and pretreatment of cells new plates were incubated with 50 and 10 µl of cell lysate. After five days six plaques were picked again and new CV-1 cells were incubated with these plaque lysates. After the first two rounds of plaque selection there was no pretreatment with MPA, xanthine and hypoxanthine. When the drugs were removed, the desired double cross-over recombinant virus without the *gpt* gene could form plaques and three more rounds of selection without the drugs followed. In these rounds the cells were incubated with 10 and 2 µl of cell lysate. After the fifth round the plaque lysates were screened for recombinant viruses by their marker gene expression.

5.1.4 Screening for marker gene expression

In the parental virus GLV-1h190 the gene encoding the β-galactosidase was inserted in the J2R locus and the *katushka* gene encoding Turbo Red FP635 protein inserted in the A56R locus. The F14.5L locus contains the gene for a *renilla* luciferase-gfp fusion protein also used for screening purposes. However, the insertion of the

microRNA let-7a complementary sequences was located between the *D4R* gene and the *D5R* gene, so all reporter genes should be functional.

Every viral plaque lysate of the fifth round of plaque selection was used for incubation with CV-1 cells seeded in 24-well plates at a density of 2.0 x 10^5 cells / well. The cells were incubated for 1 hour with 200 µl DMEM - 2 % FBS media and 10 to 20 µl viral plaque lysate, subsequently 1 ml overlay media / well was added. After incubation for 2 days at 37 ˚C and 5% CO_2 the cells were screened for the expression of β-galactosidase. The cells were washed with PBS twice and then fixed with 3.7% of paraformaldehyde for 10 minutes at room temperature. After the incubation the paraformaldehyde was aspirated and the cells were washed with PBS twice again. The staining solution was added to the cells and they were incubated at 37 ˚C and 5% CO_2 over night until stained and non-stained viral plaques could be distinguished. A positive recombinant virus would be positive for β-galactosidase (Fig. 5.1) staining like its parental strain GLV-1h190. Positive plaques would also display the expression of GFP (Fig. 5.2) and Turbo Red FP635 (Fig. 5.3).

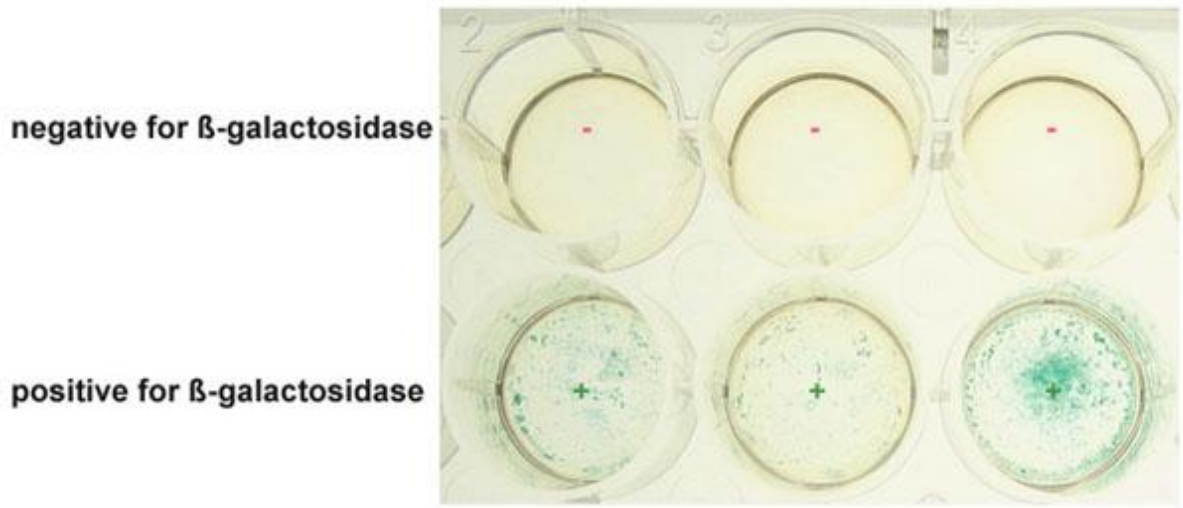


Fig. 5.1 Screening of plaque lysates by β-Galactosidase staining

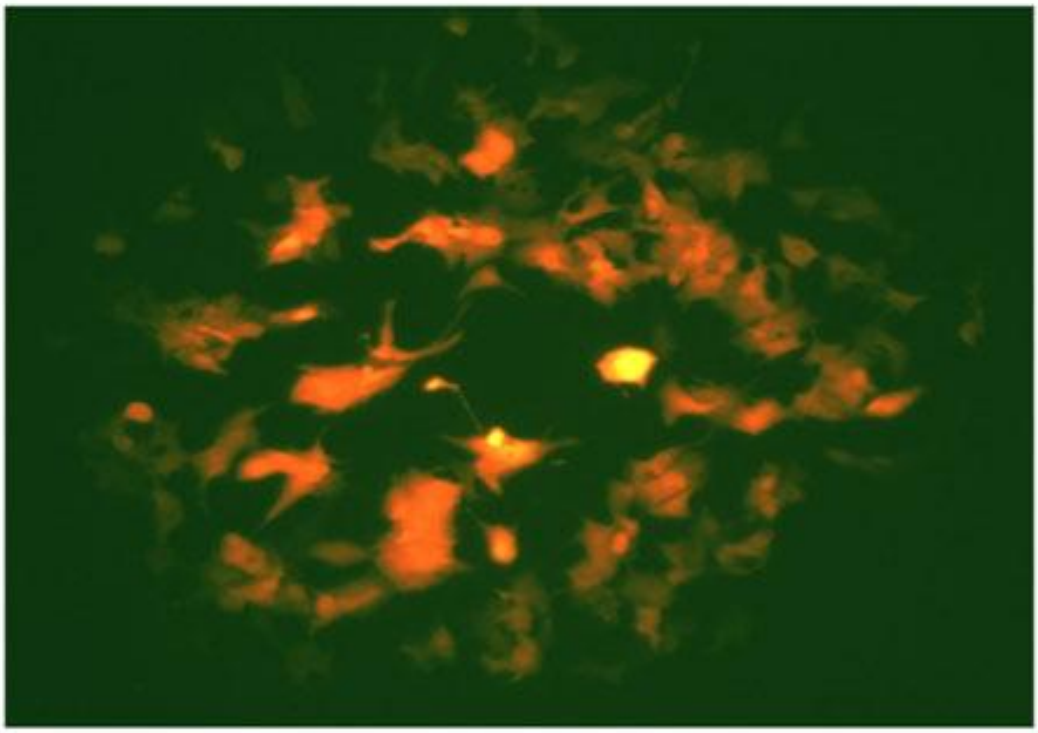


Fig. 5.2 Screening of plaque lysates for turboFP635 expression

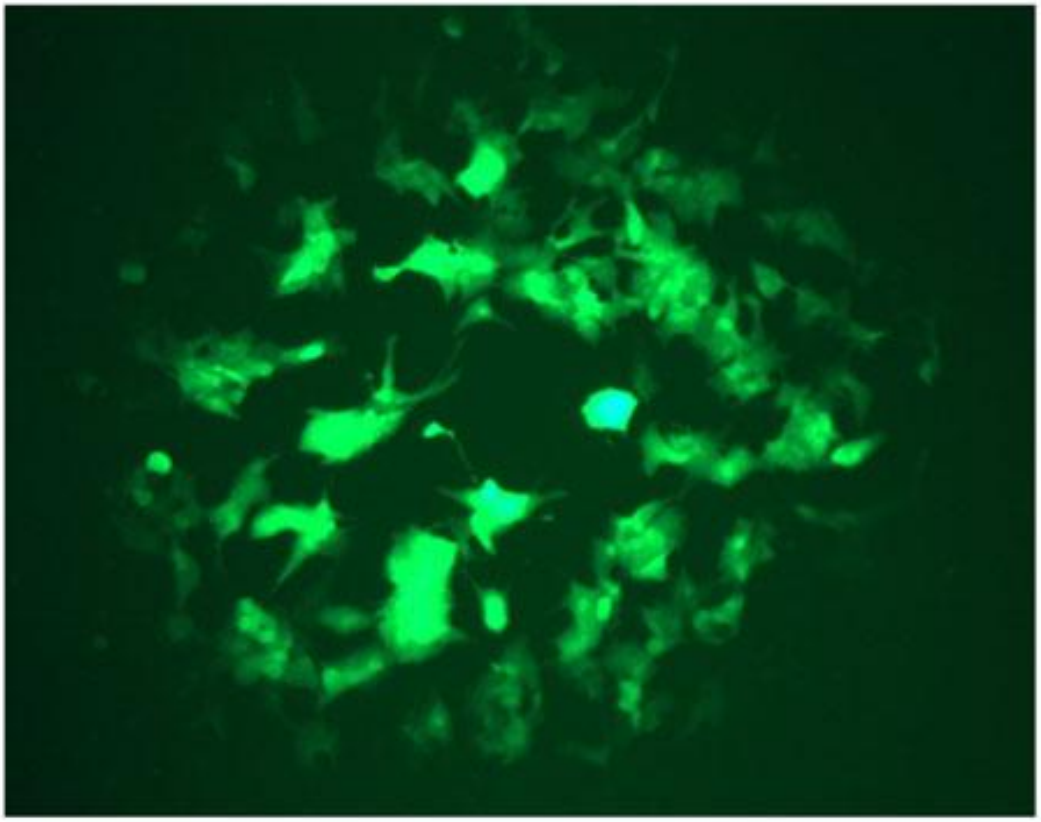


Fig. 5.3 Screening of plaque lysates for gfp expression

5.1.5 *gpt* screening

This screening was important to determine whether the recombinant virus still have the *gpt* gene inserted meaning only one cross-over event took place instead of a double cross-over.

CV-1 cells seeded in 24-well plates were treated with MPA, xanthine and hypoxanthine. After 24 hours cells were incubated with 10 µl of virus plaque lysates from the fifth round. Overlay medium was added after a one hour incubation time and plates were incubated for five days and stained with crystal violet solution. After washing the plates the wells with virus plaques could be distinguished from the wells

without plaques. The plaque lysates that don't show plaques could be used to confirm the presence of the desired gene by PCR using designed primers.

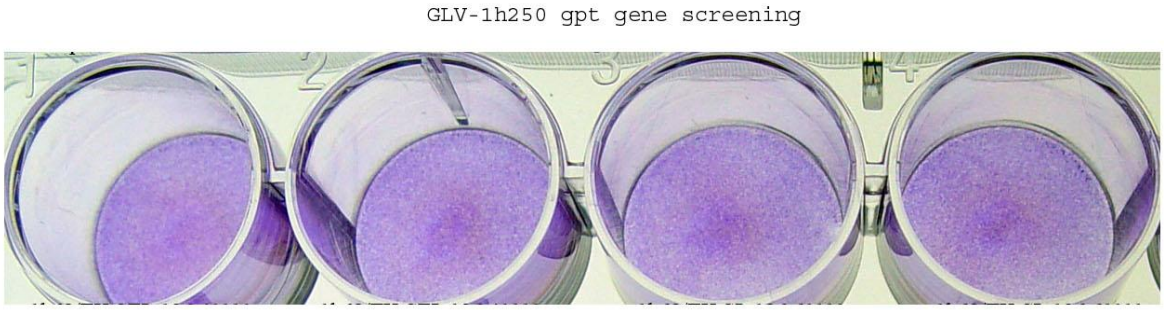


Fig. 5.4 Crystal violet staining after treatment with MPA, Xanthine and Hypoxanthine and incubation with virus plaque lysates indicates that double cross-over event has taken place resulting in missing plaque formation.

5.1.6 Isolation of DNA to verify sequence

Before amplifying the virus to create a virus stock to use for experiments, the presence and the correctness of the inserted gene has to be verified by PCR. Therefore, CV-1 cells in 60 mm dishes were incubated with 100 µl of virus plaque lysates from the fifth round that seemed to be correct after screening of marker gene expression and gpt selection. When all the cells showed virus replication, they were scraped with a cell scraper. Six hundred microliters of the lysate were used for DNA isolation. The rest of the lysate was stored at -80 ˚C as a p1-stock, which was used for the amplification of the virus. The 600 µl of the lysate were frozen and thawed three times and then 600 µl of 2x Triton X-100 buffer with Proteinase K were added. The tubes were mixed and centrifuged at 3000 rpm for five minutes. The supernatant

was transferred into a new microfuge tube and centrifuged at maximum speed for fifteen minutes. The supernatant was discarded, the pellet resuspended in 100 µl of 1xSDS buffer and incubated at 55 ˚C for 30 minutes. After incubation, the DNA was extracted twice with phenol-chloroform and the aqueous phase was saved. 1/10 volume 3.0 M sodium acetate and 2.5 volumes of 95% ethanol were added. The tube was centrifuged for fifteen minutes at top speed and the supernatant discarded. After adding 1 ml of 80% ethanol and another centrifugation step for five minutes at top speed, the supernatant was discarded and the pellet air dried. Then the pellet was resuspended in 20 µl distilled water. One microliter was used as template in a PCR reaction using the primers D4R-5 and D4R-3, the PCR product was subsequently sent out for sequencing.

5.1.7 Amplification and purification of recombinant viruses

After confirming the DNA sequence, CV-1 cells in ten T225 cell culture flasks (2×10^7 cells per flask) were incubated with 2×10^5 pfu/ml virus. The medium in the flasks was aspirated and the cells were incubated with 10 ml of virus containing medium. After two hours in a 37 ˚C and 5% CO_2 incubator and rocking the flasks every 30 minutes to ensure better distribution of the virus, 15 ml of DMEM medium containing 2% FBS were added. After a two day incubation period, the cells in the flasks were scraped into the medium with a cell scraper and pipetted into sterile 50 ml tubes. Cells were centrifuged for five minutes at 3000 rpm and the supernatant was discarded. The pellets were then resuspended in 14 ml of 10 mM TrisCl, pH 9.0. The cell suspension was homogenized with 40 strokes in a glass homogenizer with a tight pestle. After

another centrifugation step for five minutes at 3000 rpm, the supernatant was saved and the pellet was resuspended in 5 ml of 10 mM TrisCl. The supernatant was saved again and pooled with the supernatant form the earlier step. The supernatant was sonicated three times for one minute, then layered on a cushion of 17 ml of 36% sucrose in 10 mM TrisCl and centrifuged for two hours at 13,000 rpm in a HB-6 rotor, Sorvall 6 Plus Refrigerated Superspeed centrifuge. The supernatant was aspirated and discarded. The viral pellet was resuspended in 1 ml of 1 mM TrisCl, pH 9.0 and sonicated once for one minute. In centrifuge tubes, sterile 24% to 40% continuous sucrose gradients were prepared the day before by layering 6.8 ml each of 40%, 36%, 32%, 28%, and 24% sucrose in 1 mM TrisCl and stored overnight in a refrigerator. The sucrose gradients were then overlayed with 1 ml sonicated viral pellet and centrifuged for 40 minutes at 12600 rpm at 4 ˚C. The virus band was transferred into a 50 ml conical tube and saved on ice. After aspirating the remaining sucrose, the pellet was resuspended in 1 ml of 1 mM TrisCl and sonified once for one minute. Subsequently the virus solution was layered on another sucrose gradient for centrifugation. The virus band was also collected and pooled with the virus band of the first centrifugation. The two volumes of 1 mM TrisCl were added. After another centrifugation step at 4 ˚C and 13000 rpm for two hours, the viral pellet was resuspended in 1 ml of 1 mM TrisCl. Before making aliquots the virus was sonicated three times for one minute.

5.1.8 Determination of the virus titer by plaque assay

For determining the virus titer, ten-fold serial dilutions were prepared. CV-1 cells seeded in 24-well plates and duplicates were used for addition of 200 µl of the virus dilutions. After one hour of incubation 1 ml of overlay medium was added. After two days the plates were stained with 250 µl of crystal violet solution per well and stained for several hours. Virus plaques were counted.

The titer, defined as plaque forming units (pfu) / ml, was calculated with the following formula:

$$\frac{\textit{plaque forming units (pfu)} \times \textit{dilution factor}}{\textit{Volume of virus in media}} = \textit{pfu/ml}$$

5.2. Cell culture methods

In this study the following cell lines were used: CV-1 cells, the cell line used for Transfection, titrations, etc was considered the control cell line. HEK293 (human embryonic kidney fibroblasts) cells were transfected with microRNA let-7a prior to virus inoculation to simulate healthy, non-tumorous cells. ERC (human endometrial regenerative cells) cells were used for replication studies as they were considered healthy replicating cells with a naturally high expression level of microRNA let-7a. The A549 (human lung carcinoma cells) cell line was used in viral replication studies

as the positive control cell line because in this cell line the expression of microRNA let-7a is strongly downregulated.

5.2.1. Growth and maintenance of adherent mammalian cells

Growth and Maintenance of all adherent cells used in this dissertation were performed either by Terry Trevino, Uma Sukhwani, Dominique Maciejewski-Lenoir or by myself under close supervision of one of the people mentioned above. In brief, the cells were seeded in T-225 flasks at a density of 2-3 x 10^6 cells / flask and grown using 45 ml medium / flask with each cell line´s specific medium (see Materials section) at 37°C, 5% CO_2. The media were changed every 2-3 days.

5.2.2. Transient transfection of adherent mammalian cells with small RNAs

Transfection is a method by which DNA, RNA or protein may be introduced into a cultured mammalian cell. In this study HEK293 cells and A549 cells transiently transfected with naked microRNA let-7a. A transient transfection means that the introduced microRNA is being expressed within the transfected cell but is not passed on to the daughter cells during cell doubling. For the transfection reaction 50 nM final concentration of RNA was mixed with 200µl buffer 4 µl Transfection agent (both Polyplus™) and added to the cells. After 6 hours the media is changed to normal growth media and the cells were ready to use the following morning.

5.2.3. Stable transfection of adherent mammalian cells with small RNAs

A stable transfected cell expresses the introduced gene of interest and passes this gene on to its daughter cells through integration into the cell genome. A stable transfection requires the co-transfection with genes for selection e.g. a Puromycin-resistance gene. The expression of the resistance gene allows the successfully transfected cell to survive when the toxin is added to the growth media whereas cells without the resistance gene die. The result after many selection rounds is a homogenous population of cells that permanently express the gene of interest. To generate a stable hsa-miR-let-7a expressing cell line, A549 cells were seeded at 2.0 x 10^5/ml in a 6-well plate and co-transfected with pMIR-let-7a (System Biosciences), a lentiviral microRNA expression vector to produce mature microRNA let-7a when introduced into a cell and pIRES-puro (Invitrogen) in different concentrations using JetPrime™ reagent (Polyplus™) following the manufacturer's protocol. After culturing the cells for 48h the medium was changed to RPMI-10%FBS containing 1µg/ml Puromycin. The medium was changed every two days for 2 weeks. Subsequently positive clones were bulk passaged into flasks and kept under selective pressure.

5.3 Virological methods

To study the behavior of GLV-1h250 *in vitro*, different cell lines were used for viral replication studies with varying amounts of virus, and the replication efficacy was

determined by subsequent titration. CV-1 cells were utilized as producer and control cell line, HEK293 and A549 cells were transfected to establish positive controls and investigate a potential effect of transfected miRNA on these cells. In addition, ERC cells were used for viral replication studies.

5.3.1 Vaccinia virus uptake and replication in cells

Cells were seeded in 6-well cell culture plates and grown to a confluency of 95-100%. After the cells were examined under the microscope to determine the overall health of the cells, the media was aspirated from 3 wells, and 500 µl per well of Trypsin-EDTA was added. After an incubation of 8-10 minutes at 37°C and 5% CO_2, 500 µl fresh media per well was added to stop the dislodging process. The cells were pooled into a 50 ml conical tube and resuspended carefully to reach single cell state. 100 µl cell suspension was mixed with 400 µl Trypan blue solution, and 10 µl were pipette into the upper and the lower chamber of a hematocytometer. The cells of all quadrants were counted, and the cell number per ml was calculated using the following formula:

$$\frac{\textit{Average cell count per quadrant } x \textit{ } 5}{0.0001} = \textit{cells/ml}$$

The required amount of virus was calculated (see 3.1.8) based on the cell count. Prior to adding the virus suspension, virus aliquots were thawed on ice and sonified

for 30 seconds at 4 ˚C. This procedure prevented the formation of virus aggregates. The media was aspirated from the cells and the desired amount of virus was added to 500 µl of new media. After one hour of incubation at 37 ˚C and 5% CO_2 with gentle agitation every 20 minutes, fresh culture medium was added.

5.3.2 Viral replication

The viral replication assays were performed in ERC, A549 and HEK293 cells with CV-1 cells as a control cell line. The cells were seeded in 6-well plates and virus suspension was added using a multiplicity of infection (MOI) of 0.01. After one hour of incubation at 37 ˚C and 5% CO_2 with gentle agitation every 20 minutes, fresh culture medium was added. The cells were harvested after 24, 48 and 72 hours by scraping the cells off the culture plate with a cell scraper. Following three freeze-thaw cycles, serial dilutions of the lysates were titered by standard plaque assay on CV-1 cells. All samples were measured in triplicates.

5.3.3 Plaque assay

The standard plaque assay is a method to determine viral titers in a suspension. CV-1 cells were grown in 24-well-plates to 100% confluence. Samples were sonicated three times for 30 seconds and diluted depending on the expected virus titer. Then CV-1 cells were incubated with 200 µl of virus dilutions. After one hour of incubation at 37 ˚C and 5% CO_2 with gentle agitation every 20 minutes, 1 ml of

carboxymethylcellulose (CMC) overlay medium was added. The cells were incubated at 37 ˚C and 5% CO_2. Fourty eight hours later the cells were stained with 250 µl of crystal violet solution per well. Several hours after the staining, plates were washed and colorless viral plaques could be identified and counted. All samples were measured as triplicates and the results were averaged to obtain better accuracy. The final pfu/ml was calculated using the formula described in 5.1.8.

5.4. Protein analytical methods

The let-7a complementary sequence repeats are located in the 3'-ÚTR of the D4R gene. If endogenously expressed microRNA let-7a binds its target, the mRNA should be degraded and no D4R protein should be detectable. The D4R antibody used in this study was designed by Genscript. The protein sequence of D4R was sent in and the company chose an antigenic region to generate an antibody in rabbits. The D4R protein sequence and the peptide sequence are shown below:

Protein sequence D4R:

MNSVTVSHAPYTITYHDDWEPVMSQLVEFYNEVASWLLRDETSPIPDKFFIQLKQPL
RNKRVCVCGIDPYPKDGTGVPFESPNFTKKSIKEIASSISRLTGVIDYKGYNLNIIDGVI
PWNYYLSCKLGETKSHAIYWDKISKLLLQHITKHVSVLYCLGKTDFSNIRAKLESPVT
TIVGYHPAARDRQFEKDRSFEIINVLLELDNKAPINWAQGFIY

Peptide sequence Genscript:

DPYPKDGTGVPFES

5.4.1 Preparation of protein lysates from mammalian cells

Cells were grown in 6-well-plates to 90-100% confluence. The supernatant was then aspirated and the cells were washed with PBS. Then 1 ml of PBS was added to the cells and cells were scraped off the plate with a cell scraper. After centrifugation at 2.5 x g for five minutes, the cell pellet was resuspended in 100 µl of RIPA buffer with Proteinase Inhibitors. Cells were sonicated with 50% power for 30 seconds and incubated on ice for fifteen minutes. Then the lysate was treated with 1 µl benzonase and incubated for one hour at 37 ˚C to degrade the DNA. The protein concentration was determined with the D_C Protein Assay (Biorad). Therefore the exact same amount of protein could be loaded in every well of the gel which was important for comparison. Standards were prepared with concentrations from 0.2 mg/ml to 1.2 mg/ml of lyophilized Bovine Plasma Gamma Globulin (Biorad). Five microliters of standards and samples were pipetted into a 96-well microtiter plate. Twentyfive microliters of reagent A' (prepared of 20 µl of reagent S for each ml of reagent A) were added to each well. Then 200 µl of reagent B were added and the plate was mixed. After fifteen minutes, absorbance was read with a microplate reader at 750 nm, and the protein concentrations calculated according to the standard curve.

5.4.2 SDS-Polyacrylamid gel electrophoresis

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique to separate proteins according to their molecular weight. SDS is an anionic detergent that denatures proteins. It charges proteins homogenously negative by

binding to positively charged side chains of amino acids in proteins. The intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Separation of proteins is based on the amount of SDS that is bound by a protein. The SDS in the SDS-loading buffer, which is added to the protein samples prior to electrophoresis, helps degrading three-dimensional protein structures.

To perform the electrophoresis, the NuPAGE® precast gel system (Invitrogen) in an XCell SureLock™ Mini Cell (Invitrogen) was used. 10 µg of total isolated protein from each sample was mixed with SDS-loading buffer, boiled at 95 ˚C for five minutes, and centrifuged 5000 rpm for five minutes before loading a total volume of 20 µl of the supernatants onto a Precast NuPAGE® Bis-Tris (Invitrogen) gel. In the first well 10 µl of Precision Plus Protein Standard (Biorad) were loaded to compare protein sizes of the samples. The gel chamber was filled with 1x NuPAGE® MOPS Running buffer (Invitrogen). Gel electrophoresis was conducted at a voltage of 150 V for about one hour.

5.4.3 Coomassie staining of protein gels

Coomassie blue is an anionic dye, which binds with proteins non-specifically. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated in the gel could be removed by destaining with the same solution lacking the dye. The proteins would be detected as blue bands on a clear background.

After SDS-PAGE the gel was stained with Coomassie Brilliant Blue R250 Solution. The gel was incubated in Coomassie Staining solution on a shaker at room temperature. After several hours, the staining solution was removed and replaced by destaining solution. When the protein bands were clearly visible and the background destained, the gel was scanned or photographed.

5.4.4 Protein transfer to PVDF membrane (Western blot)

Proteins within a polyacrylic matrix are not accessible for macromolecular ligands like antibodies. To make them accessible to these ligands, proteins have to be transferred to a membrane. Degraded proteins can be transferred to a polyvinylidene fluoride (PVDF) membrane through migration within an ionic gradient. The negatively charged proteins migrate towards a cathode. They bind to the membrane through hydrophobic interaction.

For protein transfer, the XCell SureLock™ Mini-Cell with a blot module (Invitrogen) was used. The proteins were blotted on a 0.2 µm PVDF membrane (Invitrogen). The blot was assembled in the following order from cathode to anode: Three buffer soaked sponges, buffer soaked filter paper, polyacrylamide gel, membrane, buffer soaked filter paper, three buffer soaked sponges. The inner chamber was filled with 1x NuPAGE® Transfer buffer (Invitrogen) and the outer chamber with cold water for cooling. The proteins were blotted for 50 minutes at 35 V.

5.4.5 Colorimetric detection

After Western Blotting, the membrane was blocked with 5% skim milk for 30 minutes at room temperature on a shaker. Then it was incubated with the primary antibody with using an antibody specific dilution (anti-D4R antibody: 1:1000) in PBS over night at 4 ˚C. The membrane was washed for 30 minutes in PBST, followed by an incubation with the secondary antibody (Biorad Goat anti-Rabbit) in a 1:5000 dilution for two hours at room temperature. After a final wash of one hour in PBST, the membrane was incubated with the substrate solution from the Opit-4CN kit (Biorad) for colorimetric detection following the manufacturer's protocol until protein bands were visible.

5.4.6 Chemiluminescent detection

Because colorimetric detection failed to detect D4R, a different secondary antibody was used for chemiluminescent detection, which is more sensitive than the colorimetric detection method.

For the chemiluminescent detection the membrane was incubated with a substrate which visualizes horseradish peroxidase (HRP) bound to proteins as dark bands when exposed to x-ray film. After incubation of the secondary antibody (Biorad Goat anti-Rabbit HRP conjugated) at a concentration of 1:5000, the membrane was washed with PBS + 0.05% Tween 20 three times for 5 minutes each. The Promega ECL-Plus Western Blotting Detection Kit was used following the manufacturer's protocol using 1.5 ml substrate solution per membrane instead of 2 ml substrate

solution. In brief, 1.5 ml of solution A and 35 µl substrate were mixed, pipetted drop wise onto the membrane taking care to cover the whole membrane, and incubated for 5 minutes at room temperature. After dabbing off excess substrate solution, the membrane was placed into an exposure cassette lined with a poly project jacket. In a dark room under red light conditions, an x-ray film (5 x 7 inch) was placed on the jacket covering the membrane for various times depending on the intensity of the signal. Subsequently the film was developed and fixed using a CP1000 developer from Agfa.

5.4.7 Stripping of membranes

Stripping of membranes makes it possible to detect different proteins on membranes after Western Blotting for more than one time. After finishing the detection of one antigen, the membrane could be scanned or photographed to save the result. Then it was incubated with stripping buffer for 50 minutes at 50 ˚C in a shaking incubator set at 40 rpm. Then the membrane was washed twice in 0.05% PBST for ten minutes on an orbital shaker at room temperature. Subsequently the membrane was blocked in 5% skim milk and an anti ß-actin control antibody could be incubated.

5.5 *In vivo* studies

To determine toxicity and antitumor efficacy of the newly generated virus GLV-1h250 *in vivo*, male athymic nude mice bearing A549 lung carcinoma cells implanted subcutaneous into the right flank were injected retro orbitally with a high dose of viral particles. The behavior of GLV-1h250 *in vivo* was compared to its parental strain GLV-1h190, GLV-1h68 (the strain already used in clinical trials for human application) as a positive control and PBS injected mice as negative controls. Each of the four treatment groups consisted of 15 animals where 5 animals were sacrificed at one week, four weeks and eight weeks post viral injection. The antitumor efficacy was determined by measuring the tumors with a caliper and monitoring viral GFP expression under a UV light source twice weekly. The toxicity was determined by bodyweight measurements and examination of the animals for possible pox formation and overall health status.

5.5.1. Virus preparation for mouse injection

Mice were injected retro-orbitally with $1x10^7$ pfu in 100 µl PBS. The right amount of virus was calculated for each virus that was to be injected, and mixed with PBS to yield 100 µl total per mouse. A small amount of virus was prepared additionally to what was needed for the injections to confirm the prepared viruses titers by performing a plaque assay. One hundred microliters of virus containing PBS were injected retro orbitally using insulin syringes. The remaining virus was titrated using

CV-1 cells seeded at 2.0 x 10^5/ml in 24-well-plates to confirm the amount of virus used in the *in vivo* experiment. After one hour overlay medium was added and after two days the cells were stained with crystal violet solution. After destining the plates with water, the titer was determined.

5.5.2. Tumor monitoring

Four to six weeks old male nude mice were implanted with 5x10^6 A549 lung carcinoma cells subcutaneously on the right flank of the upper hind leg area. The viruses were injected three to four weeks after tumor implantation. One times ten to the seven plaque forming units of the virus were mixed with PBS to a volume of 100 µl and injected intravenously by retro orbital injection. After injection Tumors were measured twice a week with a digital caliper. Length, height and width of the tumors were measured and recorded. The tumor volume was calculated using the following formula:

$$\text{tumor volume} = \frac{\text{length} \times \text{width} \times (\text{height} - 5\text{mm})}{2}$$

Also the weight of each mouse was noted to keep track of any changes of body weight after tumor implantation and injection with the virus which would indicate toxicity. By holding the mice under UV light and looking at the tumors, a successful virus uptake and replication could be confirmed by the visible green GFP expression mediated by the viruses and could be photographed for the records (Fig 5.5).

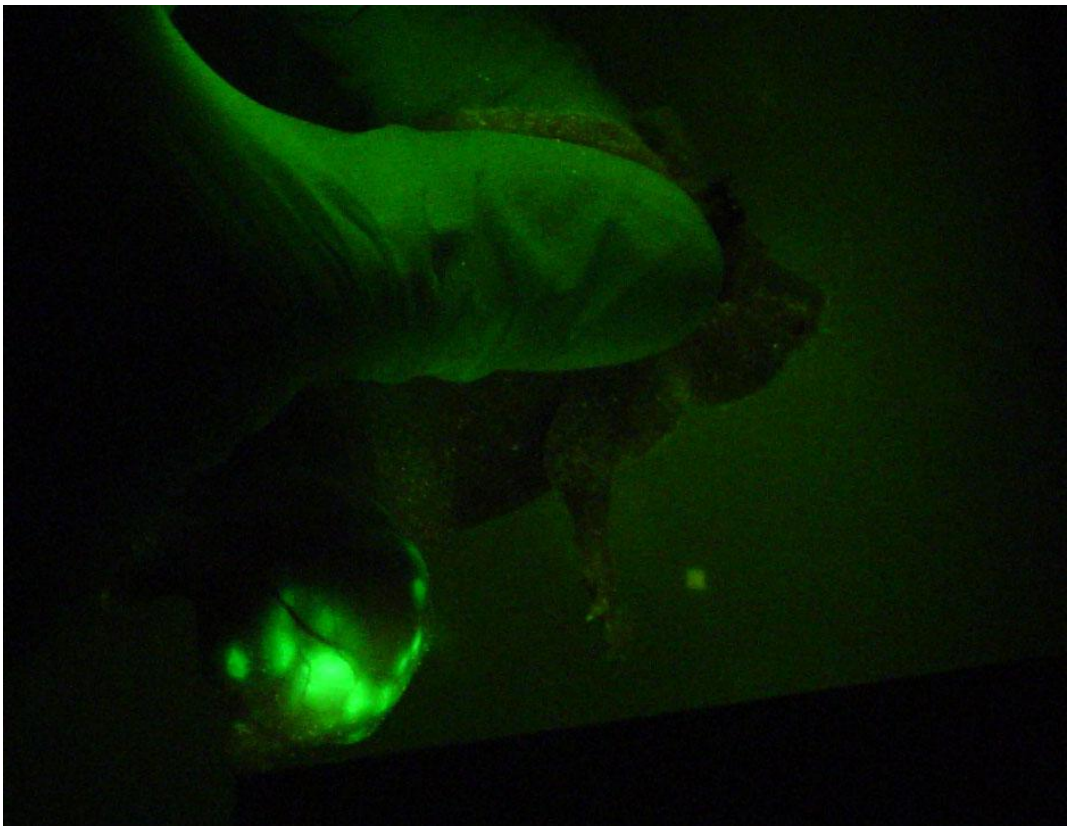


Fig 5.5 GFP expression in mouse tumor after retro-orbital injection of GLV-1h250

5.5.3. Tumor and organ preparation for virus titration

Viral toxicity was further accessed by viral biodistribution in various organs. After dissection of the tumors and organs (spleen, liver, lungs, kidneys, testis, brains), they were weighed and placed in MagNA Lyser Green Beads tubes (Roche) containing 500 µl of PBS with proteinase inhibitors. One tablet of proteinase inhibitor cocktail was previously dissolved in 50 ml of PBS. If the weight exceeded 0.8 g, additional tubes were used for the same organ. It had to be noted how many tubes were used so it could be calculated into the end result. The tubes were kept on ice at all times.

After all tumors and organs were dissected they were shredded at 3000 rpm for 30 seconds with a MagNA Lyser (Roche). Then they were frozen at -80 ˚C until the day of the titration. Samples were thawed three times, then they were sonicated three times for one minute to further break up the tissue and virus aggregates. Tissues that were split into more than one tube were combined. Then the samples were centrifuged for five minutes at 6000 rpm.

10 µl of organ lysate in 200 µl of medium was added to CV-1 cells. Tumors were diluted until 10^{-6} and the dilutions 10^{-4} – 10^{-6} were used for titration. 200 µl of the dilutions were added to CV-1 cells. After one hour overlay medium was added and after two days the cells were stained with crystal violet solution. After washing and drying the virus plaques were counted and titers were calculated. The bio distribution data were presented as pfu / organ or pfu / tumor.

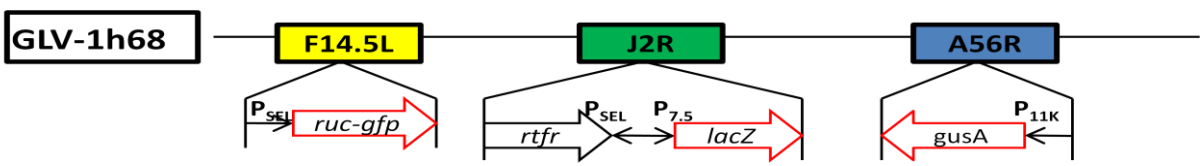
5.5 Statistical analysis

To determine the statistical relevance 5 animals of each group were taken into the analysis. Statistical analysis was conducted using student´s T test.

6 Results

6.1 Generation of Recombinant Vaccinia Virus GLV 1h250

To increase the safety and performance of GLV-1h68, a recombinant oncolytic vaccinia virus (rVACV) already in evaluation for human application in clinical trials, and other rVACV for use in laboratory settings the virus GLV-1h250 with new and different properties compared to its parental strain GLV-1h190, a derivative of GLV-1h68 was created (Figure 6.1). GLV-1h68 is an engineered replication-competent vaccinia virus which carries three reporter genes for detection purposes and a degree of attenuation suitable for clinical application. Though there are hardly any side effects reported to be found in GLV-1h68 mediated oncolytic therapy an increased tropism for replication exclusively in cancer cells is desirable. Therefore it was investigated whether or not further cancer cell specificity of a recombinant vaccinia virus strain could be obtained without compromising its oncolytic activity using microRNA interference. To achieve this goal a quadruple complementary sequence repeat of mature human microRNA let-7a was cloned behind the stop codon in the 3'end of the vaccinia virus *D4R* gene, using a GLV-1h68 derivative, GLV-1h190, as parental strain yielding the new recombinant virus GLV-1h250.



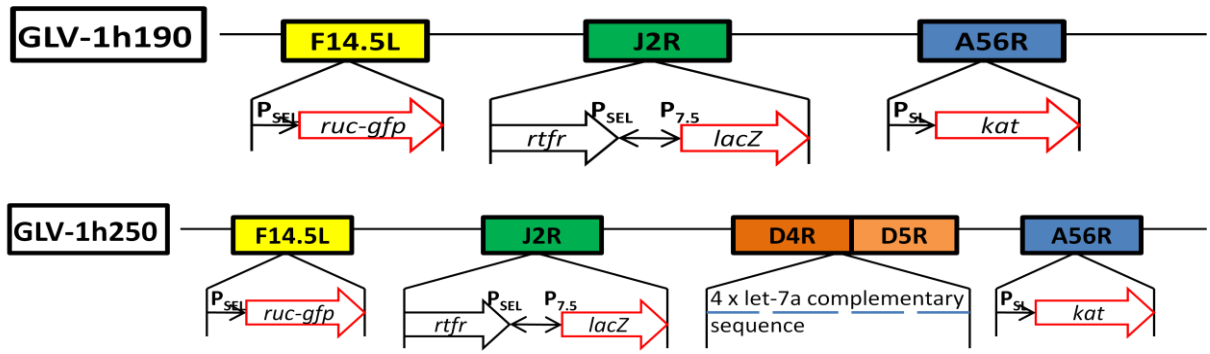


Figure 6.1: Gene maps of viruses used in this study

The gene maps display the loci used for the expression of marker genes. In case of GLV-1h250 the insertions site for the quadruple let-7a complementary sequence repeats is pointed out additionally. The expression cassette was inserted between the genes encoding the proteins D4R and D5R. The *D4R* gene contains a Flag-Tag for additional detection possibilities at its 3´end followed by the let-7a complementary repeat sequence cassette.

A transfer vector was designed to deliver the desired changes in sequence into a cell and there recombine with a virus strain chosen to be the parental backbone to create a virus carrying and expressing the introduced genes. The transfer vector pCR2.1-D3R-D4R-Flag-D5R-gpt7, designed and generated by Genelux Corporation, San Diego, CA, was used as a backbone in this study. It contained the sequences for the vaccinia virus genes *D3R*, *D4R* and *D5R*. Before the stop codon at the 3'end of the *D4R* gene the vector carried a Flag tag for detection purposes. Following the *D5R* gene the vector carried the sequence for the *E. coli gpt* gene under control of the vaccinia virus p7.5 early promoter for screening purposes.

The let-7a complementary sequence expression cassette was inserted behind the stop codon at the 3'end of the *D4R* gene to generate a recombinant virus showing a replication deficiency in healthy cells which display high expression levels of microRNA let-7a. The *D4R* gene belongs to the group of early transcribed vaccinia genes and encodes for a 25 kD small essential enzyme, uracil DNA glycosylase, which catalyzes the removal of uracil residues from double-stranded DNA, preventing

a virus defective in *D4R* from entering into the intermediate and late phase of replication under non complementing conditions. Since the uracil DNA glycosylase is crucial for vaccinia viral replication, targeting its mRNA would result in inhibition of its expression and subsequent viral replication leading to overall increased viral tropism. The unique restriction sites BamHI at the 3' end of the *D4R* gene and SpeI at the 5' end of the *D5R* gene were already present on the transfer vector and made it possible to ligate the sequence with any vector carrying the same restriction sites. Due to the length and the rather complicated secondary structure of the sequence, 4 long partially overlapping primers were designed and amplified by PCR. The first round of PCR reactions yielded two size products, one of 760 bp (D4R for / let-7a rev) and one of 617 bp (let-7a-for / D5R rev). The PCR results are displayed in figure 6.2.

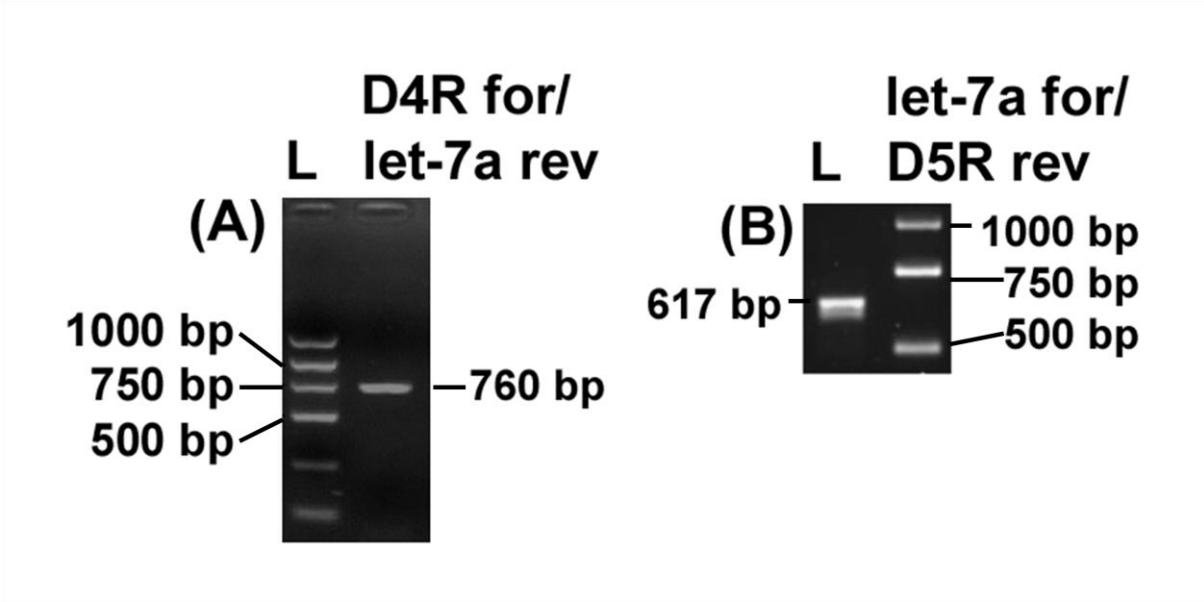


Figure 6.2.: First round PCR

The amplified DNA fragments were separated by agarose gel electrophoresis; (A) The primer pair D4R for / let-7a rev was used, L resembles a molecular ruler for size determination purposes; (B) The primer pair let-7a for / D5R rev was used, L resembles a molecular ruler for size determination.

The products of both PCR reactions were combined and used as a template for the next PCR yielding a 991 bp product (result shown in figure 6.3.).

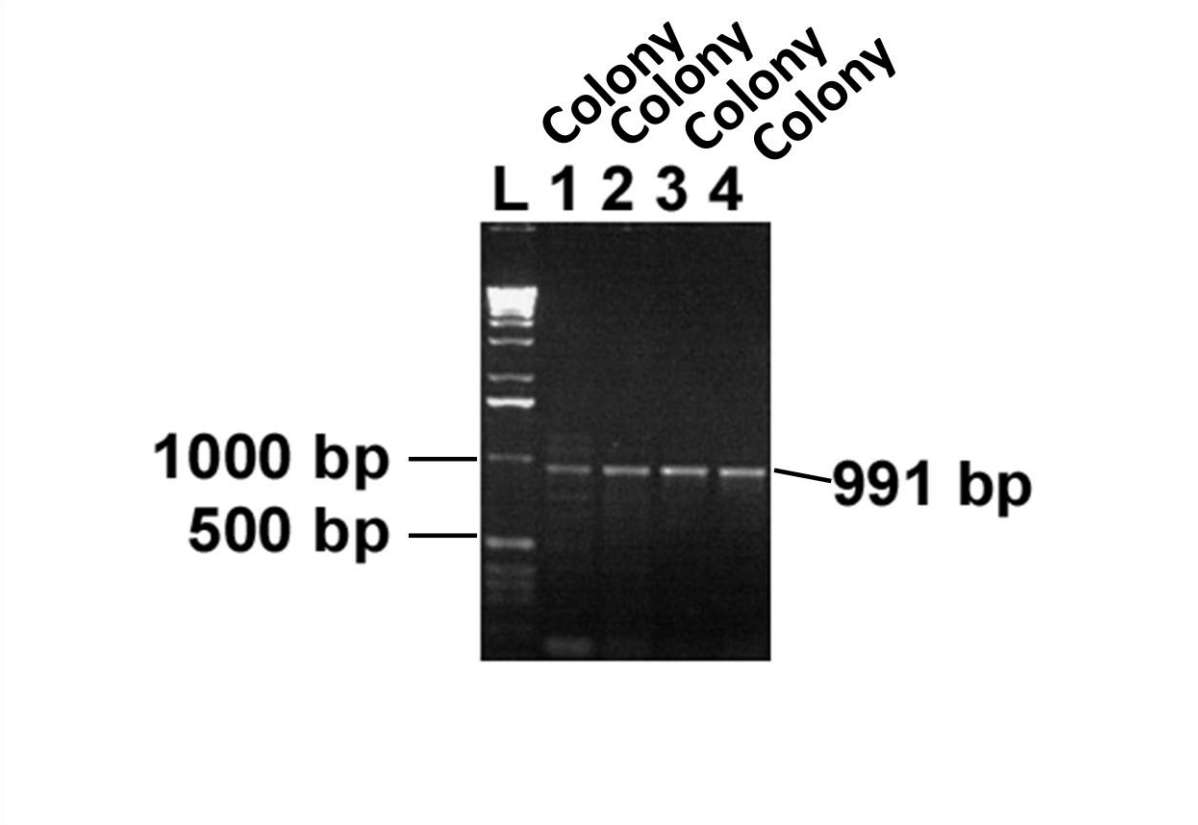


Figure 6.3.: Second round PCR

The amplified DNA fragments were separated by agarose gel electrophoresis; D4R / let-7a rev and let-7a / D5R for were used as primers yielding a 991 bp product (lanes 1-4). L resembles the molecular ruler to determine the correct size of the bands.

After the second round of PCR, the PCR product was purified using the Gel Recovery Kit (ZymoResearch) and used for a blunt-end TOPO® Cloning reaction using the pCRII®-Blunt-TOPO vector. Afterwards, the TOPO® Cloning reaction product was introduced into Top10-One Shot Chemically Competent *E. coli* (Invitrogen) using 50 µg/ml kanamycin as selective antibiotic. Plates were incubated over night and several colonies were picked for overnight bacterial cultures and subsequently used to isolate the DNA with the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) according the manufacturer's instructions. The isolated DNA was

digested with the restriction enzyme EcoRI and the clones with inserts at the expected sizes were sent out for sequencing (sequence shown in figure 6.4).

GCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTATGGATCCATGAATTCAGT
GACTGTATCACACGCGCCATATACTATTACTTATCACGATGATTGGGAACCAGTAATGAGTCAATT
GGTAGAGTTTTATAACGAAGTAGCCAGTTGGCTGCTACGAGACGAGACGTCGCCTATTCCTGATA
AGTTCTTTATACAGTTGAAACAACCGCTTAGAAATAAACGAGTATGTGTGTGTGGTATAGATCCGT
ATCCGAAAGATGGAACTGGTGTACCGTTCGAATCACCAAATTTTACAAAAAAATCAATTAAGGAGA
TAGCTTCATCTATATCTAGATTAACCGGAGTAATTGATTATAAAGGTTATAACCTTAATATAATAGA
CGGGGTTATACCCTGGAATTATTACTTAAGTTGTAAATTAGGAGAAACAAAAAGTCACGCGATTTA
CTGGGATAAGATTTCCAAGTTACTGCTGCAGCATATAACTAAACACGTTAGTGTTCTTTATTGTTTG
GGTAAAACAGATTTCTCGAATATACGGGCCAAGTTAGAATCCCCGGTAACTACCATAGTCGGATA
TCATCCAGCGGCTAGAGACCGCCAATTCGAGAAAGATAGATCATTTGAAATTATCAACGTTTTACT
GGAATTAGACAACAAGGCACCTATAAATTGGGCTCAAGGGTTTATTTATGACTACAAGGACCTAG
ACGACAAGTAAAACTATACAACCTACTACCTCAcgatAACTATACAACCTACTACCTCAaccggtAACT
ATACAACCTACTACCTCAtcacAACTATACAACCTACTACCTCAAGATCCTGCTTTAGTGAAATTTTA
ACTTGTGTTCTAAATGGATGCGGCTATTAGAGGTAATGATGTTATCTTTGTTCTTAAGACTATAGGT
GTCCCGTCAGCGTGCAGACAAAATGAAGATCCAAGATTTGTAGAAGCATTTAAATGCGACGAGTT
AGA

Figure 6.4: Sequence verification

The sequence of D4R-let7acomp-D5R verifies the results. The hsa-miR-let-7a complementary sequence is highlighted in red (4x).

A positive clone with confirmed sequence, D4R-let7acomp-D5R, was used for subcloning into the D4R transfer vector pCR2.1-D3R-D4R-D5R-gpt7 for the subsequent homologous recombination with the parental strain GLV-1h190 in CV-1 cells. Therefore both, the DNA of the insert and the vector, were digested with the

enzymes BamHI and SpeI. Since both enzymes needed different buffer compositions a sequential digest was performed. The insert DNA and the vector DNA were digested with BamHI over night at 37°C and subsequently purified using the DNA Clean and Concentrator™-Kit (Zymo Research). After purification, the insert and the vector were digested with SpeI at 37°C over night. The vector DNA was treated with CIP over night to avoid ligation of the vector with itself due to catalyzation of the removal of 5´ phosphate groups from DNA, RNA, ribo- and deoxyribonucleoside triphosphates by the alkaline phosphatase (Sambrook et al. 1989). The digested inset and vector DNA was separated by gel electrophoresis. Bands of expected were cut out and purified with the Zymoclean™ *Gel* DNA Recovery Kit (Zymo Research) and used for ligation.

Ligation was performed with the Quick T4 DNA Ligase (New England Biolabs) according to the manufacturer's instructions. After the ligation the newly created plasmid DNA was transformed into Top10-One Shot Chemically Competent *E. coli* using kanamycin as selection antibiotic and several colonies were screened before DNA isolation. A control restriction was performed using AgeI enzyme after DNA isolation, which displayed one restriction site within the let-7a complementary sequence and one outside within the *D5R* gene and yielded bands of the expected size of ~130bp (figure 6.5).

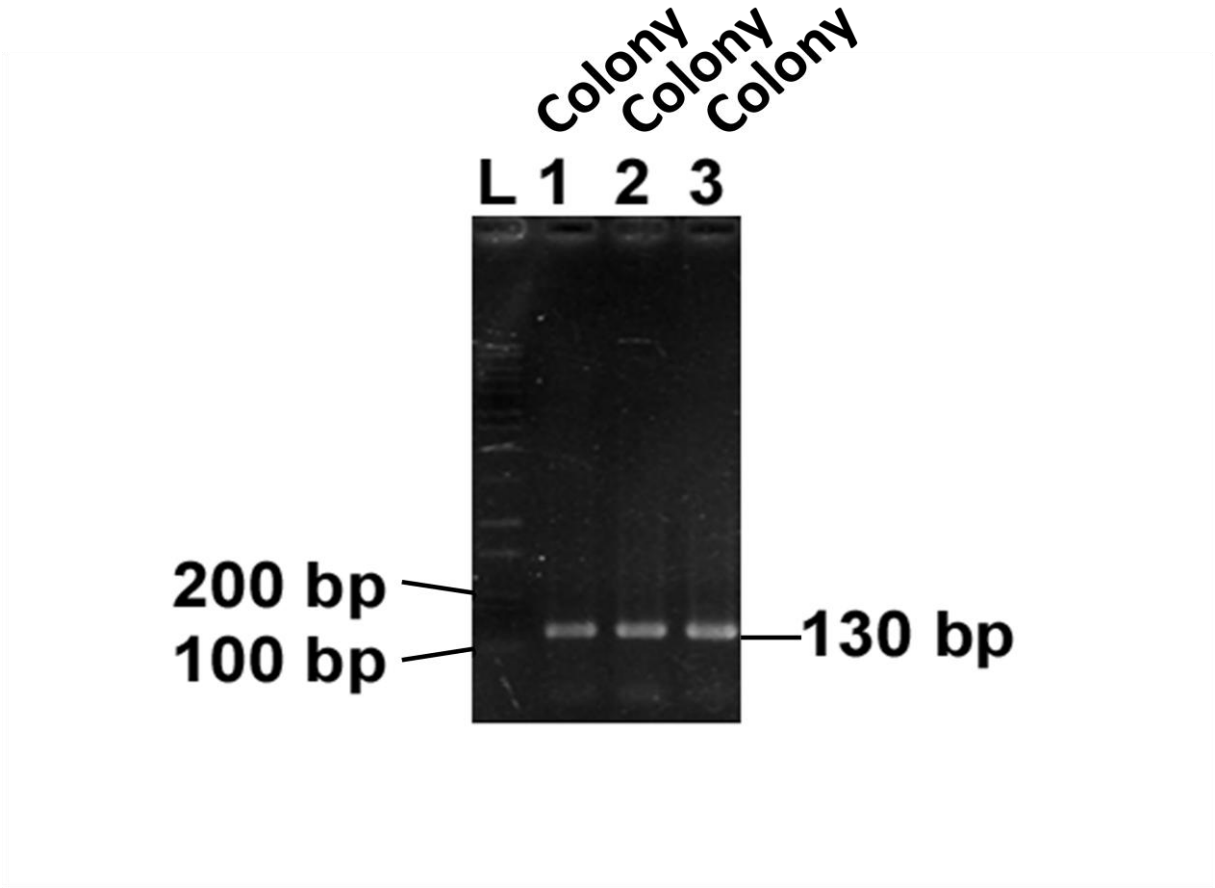


Figure 6.5.: AgeI control restriction

Control restriction of three picked colonies (lanes 1-3) with AgeI enzyme. L resembles a molecular ruler for size determination purposes.

After verifying the cut DNA sizes, the DNA of the colonies showing the right sized insertion were sent out for sequencing (figure 6.6). The sequence was verified and the successfully generated plasmid DNA was used for homologous recombination with GLV-1h190 to create a new virus.

ATAGTTAAGGANAATAAGTATCCCAAAGTCGATAACGACGATAACGAAGTATTTATACTTTTAGGA
AATCACAATGACTTTATCAGATTAAAATTAACAAAATTAAAGGAGCATGTATTTTTTTCTGAATATAT
TGTGACTCCAGATACATATGGATCTTTATGCGTCGAATTAAATGGGTCTAGTTTTCAGCACGGCG
GTAGATATATAGAGGTGGAGGAATTTATAGATGCTGGAAGACAAGTTAGATGGTGTTCTACATCC
AATCATATATCTAAAGATATACCCGAAGATATGCACACTGATAAATTTGTCATTTATGATATTTATAC

GTTTGATTCGTTCAAGAATAAACGATTGGTATTCGTACAGGTACCTCCGTCGTTAGGAGATGATAG

TCATTTGACTAATCCGTTATTGTCACCGTATTATCGTAATTCAGTAGCCAGACAAATGGTCAATGAT

ATGATTTTTAATCAAGATTCATTTTTAAAATATTTATTAGAACATCTGATTAGAAGCCACTATAGAGT

TTCTAAACATATAACAATAGTTAGATACAAGGATACCGAAGAATTAAATCTAACGAGAATATGTTAT

AATAGAGATAAGTTTAAGGCGTTTGTATTCGCTTGGTTTAACGGCGTTTCGGAAAATGAAAAGGTA

CTAGATACGTATAAAAAGGTATCTAATTTGATATAAGGATCCATGAATTCAGTGACTGTATCACAC

GCGCCATATACTATTACTTATCACGATGATTGGNAACCAGTAATGAGTCAATTGGTAGAGTTTTAT

AACGAAGTAGCCAGTTGGCTGCTACGAGACGAGACGTCGCCTATTCCTGATAAGTTCTTTATACA

GTTGAAACAACCGCTTAGAAATAAACGAGTATGTGTGTGTGGTATAGATCCGTATCCGAAAGATG

GAACTGGTGTACCGTTCGAATCACCAAATTTTACAAAAAAATCAATTAAGGAGATAGCTTCATCTA

TATCTAGATTAACCGGAGTAATTGATTATAAAGGTTATAACCTTAATATAATAGACGGGGTTATACC

CTGGAATTATTACTTAAGTTGTAAATTAGGAGAAACAAAAAGTCACGCGATTTACTGGGATAAGAT

TTCCAAGTTACTGCTGCAGCATATAACTAAACACGTTAGTGTTCTTTATTGTTTGGGTAAAACAGAT

TTCTCGAATATACGGGCCAAGTTAGAATCCCCGGTAACTACCATAGTCGGATATCATCCAGCGGC

TAGAGACCGCCAATTCGAGAAAGATAGATCATTTGAAATTATCAACGTTTTACTGGAATTAGACAA

CAAGGCACCTATAAATTGGGCTCAAGGGTTTATTTATGACTACAAGGACCTAGACGACAAGTAAA

ACTATACAACCTACTACCTCAcgatAACTATACAACCTACTACCTCAaccggtAACTATACAACCTACTA

CCTCAtcacAACTATACAACCTACTACCTCAAGATCCTGCTTTAGTGAAATTTTAACTTGTGTTCTAA

ATGGATGCGGCTATTAGAGGTAATGATGTTATCTTTGTTCTTAAGACTATAGGTGTCCCGTCAGCG

TGCAGACAAAATGAAGATCCAAGATTTGTAGAAGCATTTAAATGCGACGAGTTAGAAAGATATATT

GAGAATAATCCAGAATGTACACTATTCGAAAGTCTTAGGGATGAGGAAGCATACTCTATAGTCAGA

ATTTTCATGGATGTAGATTTAGACGCGTGTCTAGACGAAATAGATTATTTAACGGCCATTCAAGAT

TTTATTATCGAGGTGTCAAACTGTGTAGCTAGATTCGCGTTTACAGAATGCGGCGCCATTCATGAA

AATGTAATAAAATCCATGAGATCTAATTTTTCATTGACTAAGTCTACAAATAGAGATAAAACAAGTT

TTCATATTATCTTTTTAGATACGTATACCACTATGGATACATTGATAGCTATGAAACGAACACTATT

AGAATTAAGTAGATCATCTGAAAATCCACTAACCAGATCGATAGACACTGCCGTATATAGGACTAG

TAACGGCCGCCAGTGTGCTGGAATTCTAGAAAAGTAGAAAATATATTCTAATTTATTGCACGGGAT

GAGCGAAAAATACATCGTCACCTGGGACATGTTGCAGATCCATGCACGTAAACTCGCAAGCCGA

CTGATGCCTTCTGAACAATGGAAAGGCATTATTGCCGTAAGCCGTGGCGGTCTGGTACCGGGTG

CGTTACTGGCGCGTGAACTGGGTATTCGTCATGTCGATACCGTTTGTATTTCCAGCTACGATCAC

GACAACCAGCGCGAGCTTAAAGTGCTGAAACGCGCAGAAGGCGATGGCGAAGGCTTCATCGTTA
TTGATGACCTGGTGGATACCGGTGGTACTGCGGTTGCGATTCGTGAAATGTATCCAAAAGCGCAC
TTTGTCACCATCTTCGCAAAACCGGCTGGTCGTCCGCTGGTTGATGACTATGTTGTTGATATCCC
GCAAGATACCTGGATTGAACAGCCGTGGGATATGGGCGTCGTATTCGTCCCGCCAATCTCCGGT
CGCTAAGGATCAGCTTGGCGTAATCAAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGC
TCGAGCA

Figure 6.6.: Sequence verification

Sequence of pCR2.1-D3R-D4R-let-7acomp-D5R-gpt7 verifies the results. The hsa-miR-let-7a complementary sequence is highlighted in red (4x).

To achieve the homologous recombination CV-1 cells were first incubated with the virus chosen to be the parental strain followed by co-transfection with the newly generated plasmid DNA. Visible plaques were harvested and used for further plaque selection to yield a positive recombinant virus. Therefore, CV-1 cells were treated with the three drugs mycophenolic acid (MPA), xanthine, and hypoxanthine the day before incubation with the virus. MPA is an inhibitor of purine metabolism and blocks the pathway for GMP synthesis. It interferes with the replication of vaccinia and reduces the size of the virus plaques. However, this effect could be overcome by expression of the *E. coli gpt* gene in the presence of xanthine and hypoxanthine. The *gpt* gene was inserted next to the gene of interest and outside the vaccinia DNA flanking regions. Under these conditions only the single cross-over recombinant virus would express xanthine-guanine phosphoribosyltransferase (gpt), which would lead to enrichment over the parental virus (Falkner and Moss, 1990). The day after pretreatment with these drugs, the cells were incubated with different amounts of virus containing cell lysate and grown for 5 days. Each selection round six single plaques were picked by aspiration. After the first two rounds of plaque selection there

was no pretreatment with MPA, xanthine and hypoxanthine. When the drugs were removed, the desired double cross-over recombinant virus without the *gpt* gene could form plaques and three more rounds of selection without the drugs followed. After the fifth round the plaque lysates were screened for recombinant viruses by their marker gene expression. In the parental virus GLV-1h190 the gene encoding the β-galactosidase was inserted in the J2R locus and the *katushka* gene encoding Turbo Red FP635 protein inserted in the A56R locus. The F14.5L locus contains the gene for a *renilla* luciferase-gfp fusion protein also used for screening purposes. However, the insertion of the microRNA let-7a complementary sequences was located between the *D4R* gene and the *D5R* gene, so all reporter genes should be functional.

Every viral plaque lysate of the fifth round of plaque selection was screened for the expression of β-galactosidase. Therefore the cells were fixed with paraformaldehyde and incubated with the staining solution until stained and non-stained viral plaques could be distinguished. A positive recombinant virus would be positive for β-galactosidase staining like its parental strain GLV-1h190. Positive plaques would also display the expression of GFP and Turbo Red FP635. The plaques number 11111, 12111 and 13111 displayed positive ß-galactosidase staining results and were positive for GFP and Turbo Red FP635 expression as demonstrated in figures 6.7.-6.9.

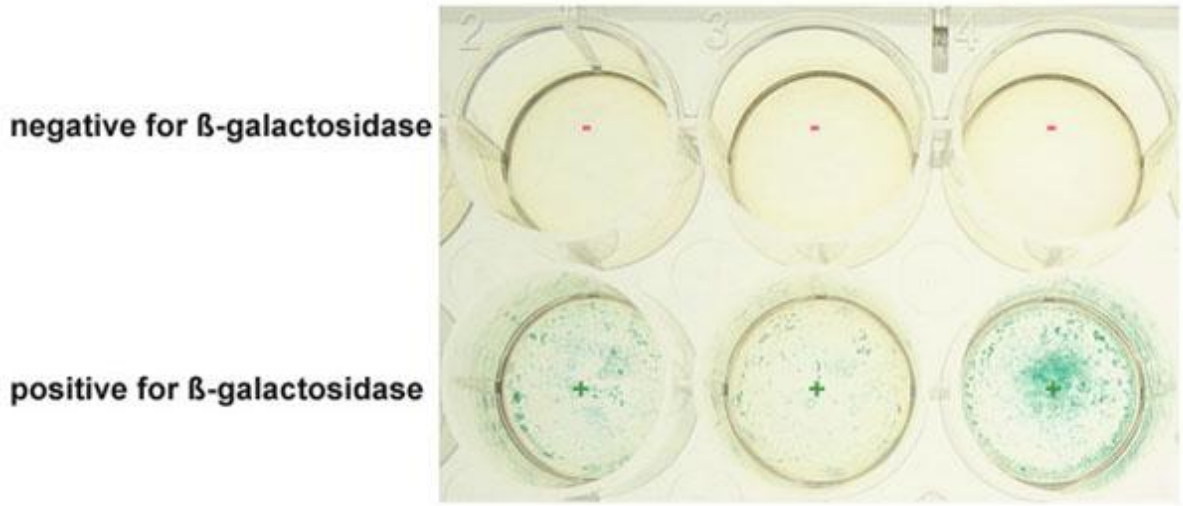


Fig. 6.7.: Screening of plaque lysates by β-galactosidase staining

The cells were fixed with paraformaldehyde and subsequently incubated with x-gal at 37°C, 5% CO_2 over night. The lower row of wells shows the plaques 11111, 12111 and 13111 (left to right) which display blue color reaction after exposure to the substrate.

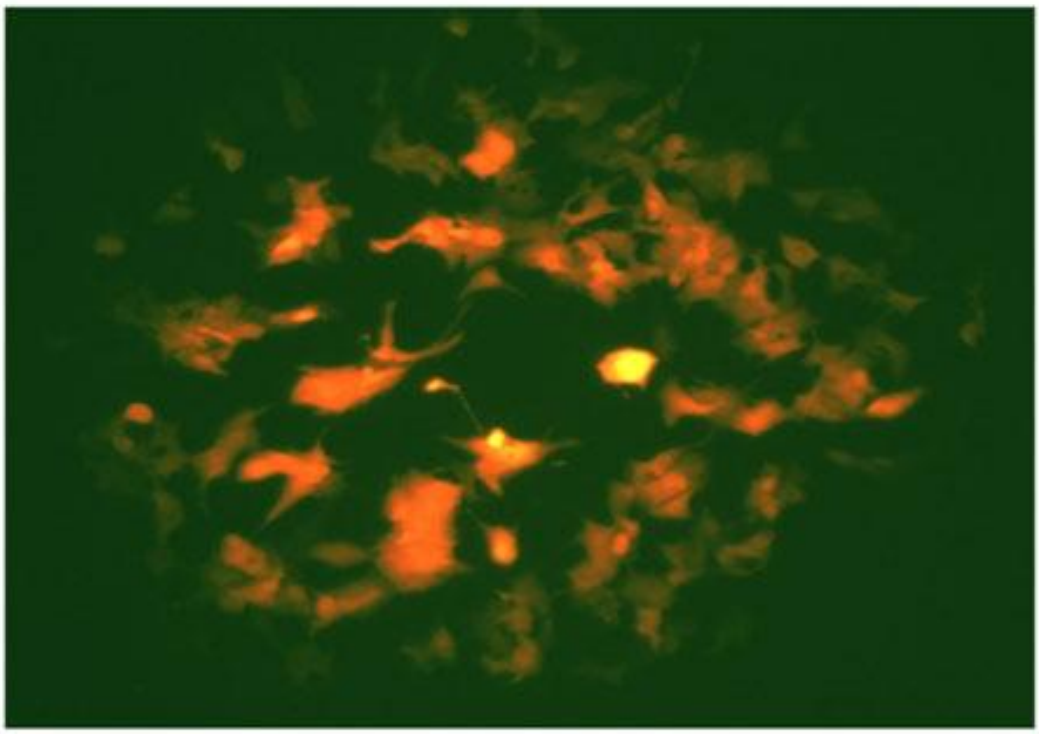


Fig. 6.8.: Screening for Turbo Red FP635 expression in CV-1 cells incubated with virus plaque lysate

Turbo Red FP635 expression in CV-1 cells incubated with plaque lysate 11111 using a fluorescent microscope set to 10x magnification. The image was taken 24 hours post incubation with 10 µl viral plaque lysate.

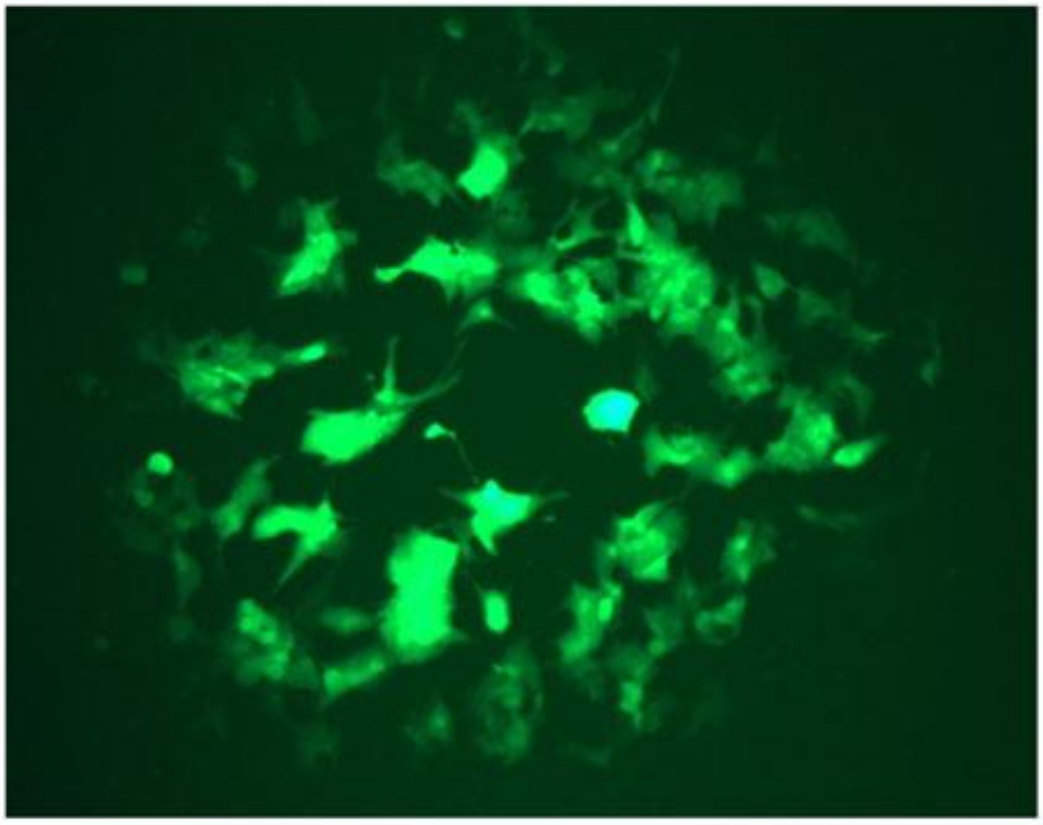


Fig. 6.9.: Screening for GFP expression in CV-1 cells after addition of virus plaque lysate

Gfp expression in CV-1 cells incubated with plaque lysate 11111 using a fluorescent microscope set to 10x magnification. The image was taken 24 hours post incubation with 10 µl viral plaque lysate.

To determine whether the recombinant virus still had the *gpt* gene insertion, a screening of fifth round viral plaque lysates was conducted. Viruses able to replicate in the presence of mpa/xanthine/hypoxanthine would be considered single cross-over viruses while the desired double cross-over event would be depicted by complete replication inhibition of the virus in presence of the three drugs (figure 6.10).

The plaque lysates that did not show plaques could be used to confirm the presence of the inserted complementary microRNA sequence cassette by PCR using the primers D4R-5 and D5R-4.

Figure 6.10.: Crystal violet staining of pretreated CV-1 cells after incubation with virus plaque lysates

CV-1 cells were treated with MPA, Xanthine and Hypoxanthine and incubated with viral plaque lysates, 11111, 12111, 13111 and 14111 (left to right).

Before amplifying the virus to create a virus stock to use for experiments, the presence and the correctness of the inserted gene was verified by PCR. Therefore, CV-1 cells were incubated with virus plaque lysates from the fifth round found to be correct after screening of marker gene expression and gpt selection. When all the cells showed virus replication indicated by total cytopathic effect, they were harvested with a cell scraper. 600 µl of the lysate were used for DNA isolation after three freeze/thawing cycles followed by DNA recovery using phenol-chlorophorm extraction. The extracted DNA was resuspended in distilled water and one microliter was used as template in a PCR reaction using the primers D4R-5 and D4R-3. The PCR product was subsequently sent out for sequencing with the results shown in figure 6.11.

>1h190let7c12111;D3RD4Rlet7D5R-2-D4R-5

NNNNNNNNNNNNNNNTANACTATTACTTATCACGATGATTGGGAACCAGTAATGAGTCAATTGGT

AGAGTTTTATAACGAAGTAGCCAGTTGGCTGCTACGAGACGAGACGTCGCCTATTCCTGATAAGT

TCTTTATACAGTTGAAACAACCGCTTAGAAATAAACGAGTATGTGTGTGGTATAGATCCGTATC

CGAAAGATGGAACTGGTGTACCGTTCGAATCACCAAATTTTACAAAAAAATCAATTAAGGAGATAG

CTTCATCTATATCTAGATTAACCGGAGTAATTGATTATAAAGGTTATAACCTTAATATAATAGACGG
GGTTATACCCTGGAATTATTACTTAAGTTGTAAATTAGGAGAAACAAAAAGTCACGCGATTTACTG
GGATAAGATTTCCAAGTTACTGCTGCAGCATATAACTAAACACGTTAGTGTTCTTTATTGTTTGGG
TAAAACAGATTTCTCGAATATACGGGCCAAGTTAGAATCCCCGGTAACTACCATAGTCGGATATCA
TCCAGCGGCTAGAGACCGCCAATTCGAGAAAGATAGATCATTTGAAATTATCAACGTTTTACTGGA
ATTAGACAACAAGGCACCTATAAATTGGGCTCAAGGGTTTATTTATGACTACAAGGACCTAGACGA
CAAGTAAAACTATACAACCTACTACCTCAcgatAACTATACAACCTACTACCTCAaccggtAACTATACA
ACCTACTACCTCAtcacAACTATACAACCTACTACCTCAAGATCCTGCTTTAGTGAAATTTTAACTTG
TGTTCTAAATGGATGCGGCTATTAGAGGTAATGATGTTATCTTTGTTCTTAAGACTATAGGTGTCC
CGTCAGCGTGCANACNAAATGAANATCCAAGATTTGTAGAAGCATTTAAATGCGACGAGTTAGAA
AGATATATTGAGAANAANCCAGAATGTACNCTANTTCGAAAGTCTTANGNATGAGGNAAGCATACT
CTATAGNCAGAATTTTCATGGATGTANATTTAGNNGCGTGNCTAGNCNAAATANATTATTNAACGG
NCNNTTCANANTTTATNATCGANGGTGGTCAAACNGNGNANCTAGAATTCGNNTNACNNNAATGN
NGNCGCCNTTNNNN

Figure 6.11.: Sequence results verifying positive homologous recombination

The hsa-miR-let-7a complementary sequence is highlighted in red (4x).

The sequencing results could confirm successful homologous recombination demonstrated by the presence of the integrated plasmid DNA in VACV GLV-1h190s derivative strain after replication in CV-1 cells. After confirming the DNA sequence, the virus was named GLV-1h250 and amplified for further characterization *in vitro* and *in vivo*. Therefore, CV-1 cells were incubated in ten T225 cell culture flasks with $2x10^5$ pfu/ml virus. After total CPR was visible, the virus was harvested and purified. The recovered viral pellet was resuspended in 1 mM TrisCl and sonified three times for one minute before making aliquots the virus. For determining the virus titer, ten-

fold serial dilutions were prepared and duplicates of CV-1 cells were incubated with the virus dilutions. After two days the plates were stained with crystal violet solution for several hours. Virus plaques were counted and the titer was calculated.

The design and cloning of the DNA fragment, the subcloning into the transfer vector and the homologous recombination with GLV-1h190 in CV-1 cells yielding the new recombinant vaccinia virus GLV-1h250 could be successfully demonstrated. A summary of the analysis performed to validate the generation of the new virus are shown below in figure 6.12. GLV-1h250's toxicity and oncolytic potential were subsequently investigated in comparison to its parental strain and GLV-1h68 *in vitro* and *in vivo*.

Plaque	**GFP**	**Turbo Red FP-635**	**x-Gal**	**x-Glc**	**postive control**	***gpt* screening**	**PCR**	**Sequencing**
11111	+	+	+	-	+	-	+	+
12111	+	+	+	-	-	-	-	-
13111	+	+	+	-	+	+	-	-
21111	+	+	+	-	+	+	-	-
22111	+	+	+	-	+	+	-	-
61111	+	+	+	-	+	+	-	-

Figure 6.12.: Summary of screening procedures to verify the generation GLV-1h250

Requirements which need to be met in order to determine the successful generation of the new recombinant vaccinia virus GLV-1h250 are highlighted in yellow.

6.2 D4R expression in cells incubated with GLV-1h250

The insertion of the let-7a complementary sequence repeats is located in the 3'-UTR of the *D4R* gene. If endogenously expressed microRNA let-7a binds its target, the mRNA should be degraded and no D4R protein (25 kD) should be detectable. The D4R antibody used in this study was designed by Genscript. The protein sequence of D4R was sent in and the company chose an antigenic region to generate an antibody in rabbits. The D4R protein sequence and the peptide sequence are shown in figure 6.13.

Protein sequence D4R:

MNSVTVSHAPYTITYHDDWEPVMSQLVEFYNEVASWLLRDETSPIPDKFFIQLKQPLRNKRV
CVCGIDPYPKDGTGVPFESPNFTKKSIKEIASSISRLTGVIDYKGYNLNIIDGVIPWNYYLSCKL
GETKSHAIYWDKISKLLLQHITKHVSVLYCLGKTDFSNIRAKLESPVTTIVGYHPAARDRQFEK
DRSFEIINVLLELDNKAPINWAQGFIY

Peptide sequence Genscript:

DPYPKDGTGVPFES

Figure 6.13.: Amino acid sequence of the VACV D4R protein and the antigenic domain for the prospected antibody designed by Genscript

The protein sequence of the vaccinia virus uracil DNA glycosylase (D4R) and the calculated peptide sequence for the antibody generation

To investigate the successful inhibition of D4R production due to a strong downregulation in viral replication protein gel analysis were performed. SDS-PAGE

(sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique to separate proteins according to their molecular weight. SDS is an anionic detergent that denatures proteins. It charges proteins homogenously negative by binding to positively charged side chains of amino acids in proteins. The intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Separation of proteins is based on the amount of SDS that is bound by a protein. The SDS in the SDS-loading buffer, which is added to the protein samples prior to electrophoresis, helps degrading three-dimensional protein structures First, ERC cells incubated with GLV-1h190, GLV-1h250 or GLV-1h68 were harvested at different time points, the cells were then lysed and the total protein was isolated. After determination of the protein concentration the exact same amount of protein could be loaded in every well of the gel.

To perform the electrophoresis, the NuPAGE® precast gel system (Invitrogen) in an XCell SureLock™ Mini Cell (Invitrogen) was used and 10 µg of total isolated protein from each sample was loaded onto the gel. To achieve an overview about the success of running the gel, it was stained with Coomassie blue. Coomassie blue is an anionic dye, which binds proteins non-specifically. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated in the gel could be removed by destaining with the same solution lacking the dye. The proteins would be detected as blue bands on a clear background (figure 6.14).

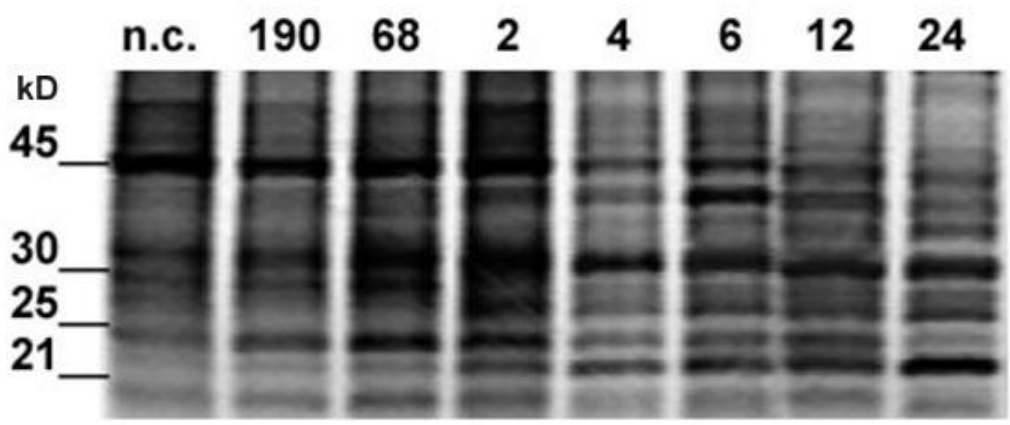


Figure 6.14.: Coomassie blue staining of total protein isolated from ERC cells after separation by SDS-PAGE

n.c. resembles cells without virus harvested at 24 h.p.i.; 190 and 68 resemble cells incubated with GLV-1h190 and GLV-1h68 cells harvested at 24 h.p.i.; 2,4,6,8,12,24 resemble ERC cells incubated with GLV-1h250 harvested at 2, 4, 6, 8, 12 and 24 h.p.i.; the size of the D4R protein is 25 kD, the size of ß-actin used as internal control is 45 kD.

To specify and validate the correct size bands visible in the Coomassie blue staining is the protein of interest, D4R, a Western blot analysis was conducted. Proteins within a polyacrylic matrix are not accessible for macromolecular ligands like antibodies. To make them accessible to these ligands, proteins have to be transferred to a membrane. Degraded proteins can be transferred to a polyvinylidene fluoride (PVDF) membrane through migration within an ionic gradient. The negatively charged proteins migrate towards a cathode. They bind to the membrane through hydrophobic interaction.

The XCell SureLock™ Mini-Cell with a blot module (Invitrogen) was used for the transfer where the proteins were blotted on a PVDF membrane (Invitrogen). After Western blotting, the membrane was blocked with 5% skim milk and then incubated with the primary antibody with using an antibody specific dilution (anti-D4R antibody: 1:1000) in PBS over night at 4 ˚C. The membrane was washed in PBST, followed by incubation with the secondary antibody (Biorad Goat anti-Rabbit) in a 1:5000 dilution.

After a final wash in PBST, the membrane was incubated with the substrate solution from the Opit-4CN kit (Biorad) for colorimetric detection following the manufacturer's protocol. Unfortunately no protein bands could be visualized possibly due to the low sensitivity of the colorimetric detection method.

Because the colorimetric approach failed to detect D4R, a different secondary antibody was used for chemiluminescent detection, which is more sensitive than the colorimetric detection method. For the chemiluminescent detection the membrane was incubated with a substrate which visualizes horseradish peroxidase (HRP) bound to proteins as dark bands when exposed to x-ray film. After incubation of the secondary antibody (Biorad Goat anti-Rabbit HRP conjugated) at a concentration of 1:5000, the membrane was washed with PBS + 0.05% Tween 20. The Promega ECL-Plus Western Blotting Detection Kit was used following the manufacturer's protocol. After preparation the membrane was placed into an exposure cassette lined with a poly project jacket. In a dark room under red light conditions, an x-ray film (5 x 7 inch) was placed on the jacket covering the membrane for various times depending on the intensity of the signal. Subsequently the film was developed and fixed using a CP1000 developer from Agfa (results figure 6.15.). The film was incubated with the membrane over three days but did not yield clearly visible bands as seen in figure 6.15. Binding of the antibody was very weak and occurred in all samples, the negative control as well, suggesting unspecific binding of the antibody. This is possible due to the sequence within the D4R protein chosen as template for the antibody design leading to non optimal binding conditions within the method chosen for detection, the Western Blot. Possibly this antibody could be utilized successfully using the ELISA method for protein detection and quantification.

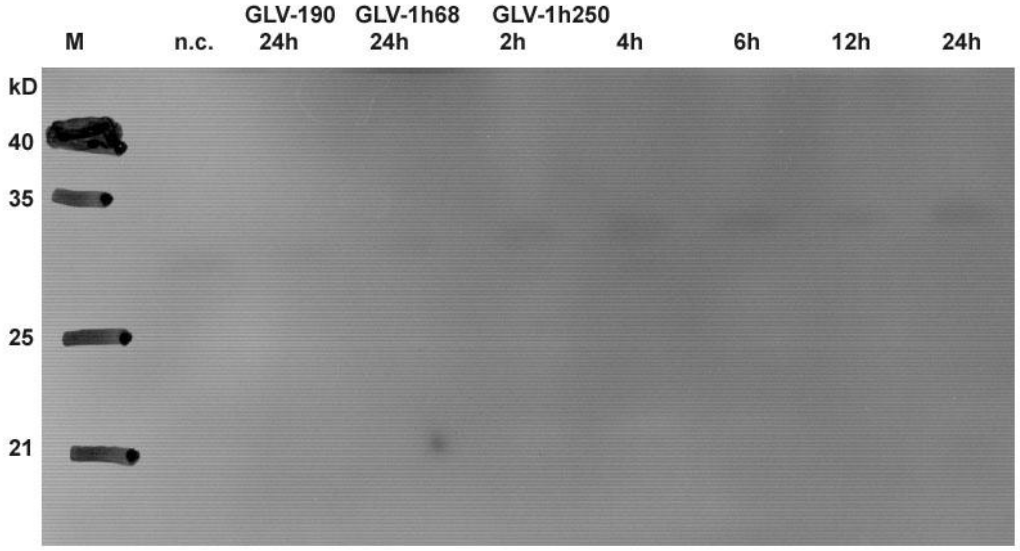


Figure 6.15.: Western blot of ERC cell lysates after incubation with anti-D4R antibody

Western blot of ERC cells incubated with virus, lysed and incubated with anti-D4R antibody. The membrane was incubated with anti-D4R antibody: 1:1000) in PBS over night at 4 ˚C. The membrane was washed in PBST, followed by incubation with the secondary antibody (Biorad Goat anti-Rabbit HRP conjugated) in a 1:5000 dilution.

To verify equal amounts of protein used in this study the membranes were stripped and screened for equal ß-actin concentrations in the samples. Stripping of membranes makes it possible to detect different proteins on membranes after Western blotting for more than one time. After finishing the detection of one antigen, the membrane could be scanned or photographed to save the result. Then it was incubated with stripping buffer and washed before blocking and incubation with an anti ß-actin control antibody. ß-actin is considered to be expressed in equal amounts within cells and can therefore be utilized as internal control (figure 6.16).

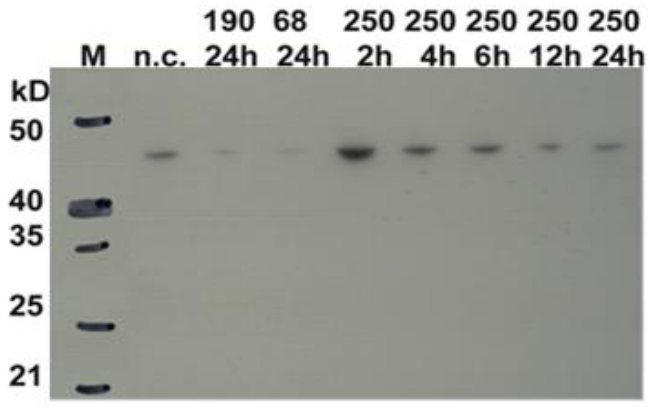


Figure 6.16.: Western blot of the ß-actin control

Western Blot of ERC cells incubated with virus, lysed and incubated with internal control antibody anti-ß-actin. The membrane was incubated with anti-ß-actin antibody: 1:1000) in PBS over night at 4 ˚C. The membrane was washed in PBST, followed by incubation with the secondary antibody (Biorad Goat anti-Rabbit HRP conjugated) in a 1:5000 dilution.

Due to varying amounts of the internal control ß-actin, as shown in figure 6.16., it has to be assumed that different amounts of sample were used in this experiment due to deviation in the pipetting process. Furthermore, the chemiluminescent detection with the anti-D4R antibody showed weak binding for all samples, including the negative control suggesting unspecific binding of the antibody. Therefore, the results obtained from the Western blot should be considered rather unreliable and the experiment should be repeated in ERC cells and additionally performed in A549 cells to ensure that microRNA mediated posttranscriptional gene silencing does not occur in cancer cells. Solid oncolytic potential could be demonstrated *in vitro* growth curves (6.3.) using A549 cells in comparison to ERC cells where the replication of GLV-1h250 was strongly decreased as well as *in vivo* when administered in mice bearing A549 lung adenocarcinoma where only the tumor was colonized by GLV-1h250.

6.3 Oncolytic potential and reduced toxicity of GLV-1h250 in cell cultures

Different cell lines were used to study the behavior of GLV-1h250 in culture. Replication studies with varying amounts of virus were conducted and the replication efficacy was determined by titration. CV-1 cells, the cell line used for transfection, titrations, etc was considered the control cell line. HEK293 (human embryonic kidney fibroblasts) and A549 (human lung carcinoma cells) cells were transiently transfected with naked microRNA let-7a prior to incubation with the virus to simulate healthy, non-tumorous cells. A transient transfection means that the introduced microRNA is being expressed within the transfected cell but is not passed on to the daughter cells during cell doubling. For the transfection reaction RNA was mixed with Transfection agent (Polyplus™) and added to the cells. After 6 hours the transfection media was changed to normal growth media. Unfortunately the transfected A549 cells did not yield enough viable cells for further replication experiments. This might be due to optimization issues and should be repeated using a microRNA-let-7a carrier linked to a gfp-reporter for an easier evaluation of successful transfection. The transfected HEK293 cells also displayed a rather low yield of viable cells. Therefore, the generated transfected HEK293 cells were used to titrate the newly generated virus GLV-1h250 in relation to its parental strain GLV-1h190 to determine a possible trend of viral replication in healthy cells. The titration in the transfected HEK 293 cell line showed a higher viral titer of the parental strain GLV-1h190 in comparison with GLV-1h250, suggesting a potential replication inhibition of the newly created virus (figure

6.16.). To verify this result the viral replication was studied in A549 adenocarcinoma cells and human ERC cells.

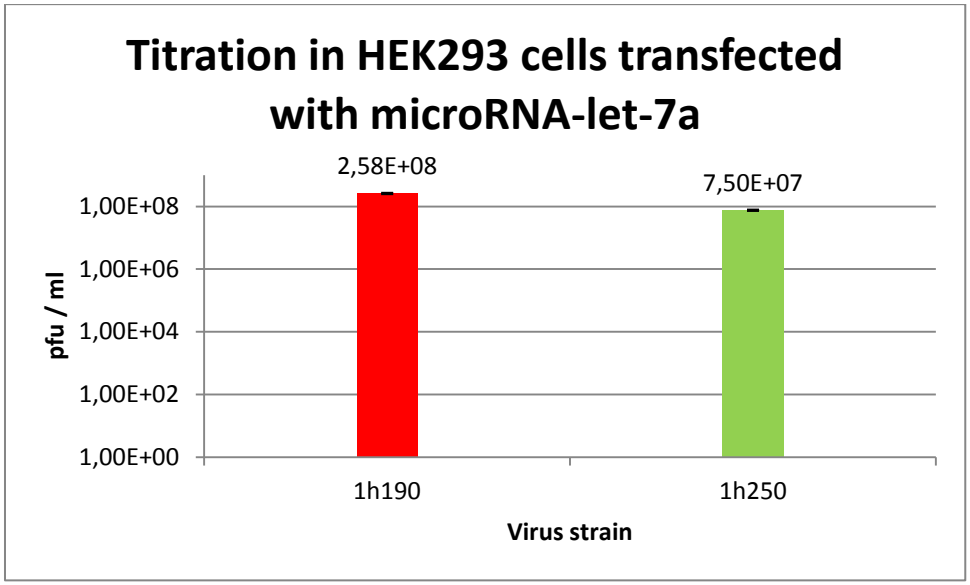


Figure 6.16.: Titration of GLV-1h190 and GLV-1h250 in transfected HEK293 cells

Titration of GLV-1h190 and GLV-1h250 in HEK293 cells transiently transfected with “naked” microRNA-let-7a.

The A549 cell line was then further used for stable transfection with microRNA-let7a to yield a genuine “control” cell line opposing the untransfected A549 cell line. Stably transfected cells express the introduced gene of interest and pass this gene on to their daughter cells through integration into the cell genome. A stable transfection requires the co-transfection with genes for selection e.g. a puromycin-resistance gene. The expression of the resistance gene allows the successfully transfected cell to survive when the toxin is added to the growth media whereas cells without the resistance gene die. The result after many selection rounds is a homogenous population of cells that permanently express the gene of interest. To generate a stable hsa-miR-let-7a expressing cell line, A549 cells were seeded at 2.0 x 10^5/ml in a 6-well plate and co-transfected with pMIR-let-7a (System Biosciences), a lentiviral microRNA expression vector to produce mature microRNA let-7a when introduced into a cell and pIRES-puro vector (Invitrogen) in different concentrations using

JetPrime™ reagent (Polyplus™) following the manufacturer's protocol. After culturing the cells for 48h, the medium was changed to RPMI-10% FBS containing 1 µg/ml puromycin. The selective medium was changed every two days. Unfortunately the transfected cells did not survive the selective pressure treatment and died after approximately 12 weeks before enough stable transfected cells could be accumulated and expanded for further experiments. A different transfection setting should be chosen in order to repeat this step through optimizing the transfection reaction, the media and growing conditions.

ERC (human endometrial regenerative cells) cells were used for replication studies as they were considered healthy replicating cells with a naturally high expression level of microRNA let-7a. Additionally, the A549 cell line was used in replication studies as the positive control cell line due to a strong downregulation of microRNA let-7a expression in this cell line. Let-7a is a well characterized microRNA known to be expressed in high levels in healthy tissues and strongly downregulated in most cancers. After viral expression of the complementary sequence, endogenously expressed microRNA-let-7a recognizes its target structure on the viral mRNA transcript and subsequently binds and degrades the viral mRNA which leads to a strong replication inhibition of the virus in healthy cells. This effect was observed when GLV-1h250 was used in replication studies with ERC and compared to its parental strain GLV-1h190 and GLV-1h68. The replication was measured by harvesting the cells over a time course of 96 hours followed by subsequent titration to determine viral titers at each time point. GLV-1h250 displayed a 10-fold reduction in viral replication rate when compared with GLV-1h190 and GLV-1h68. This could be observed whether a low Multiplicity of Infection (MOI) of 0.01 or a high MOI of 10 was used suggesting successful inhibition of viral replication in healthy cells (figures 6.17

and 6.18). Furthermore, captured pictures of the cells under a fluorescent microscope at each time point during the experiment revealed that GLV-1h250 displayed less gfp expression within and showing an overall increased health status of the cells challenged with GLV-1h250 in relation to GLV-1h68 and GLV-1h190 validating the titration data and emphasizing a decreased replication and lytic activity of the virus in healthy cells (figure 6.19).

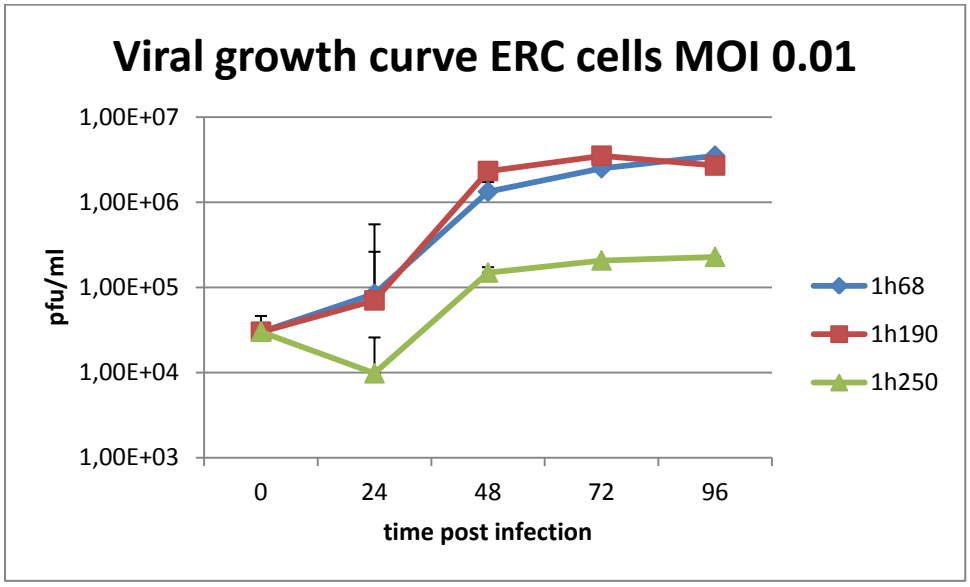


Figure 6.17.: Effect of endogenous microRNA let-7a on rVACV replication

Growth curve in ERC cells using a low MOI of 0.01. The cells were harvested and subsequently titrated in CV-1 cells to determine viral concentration within the cells at each time point.

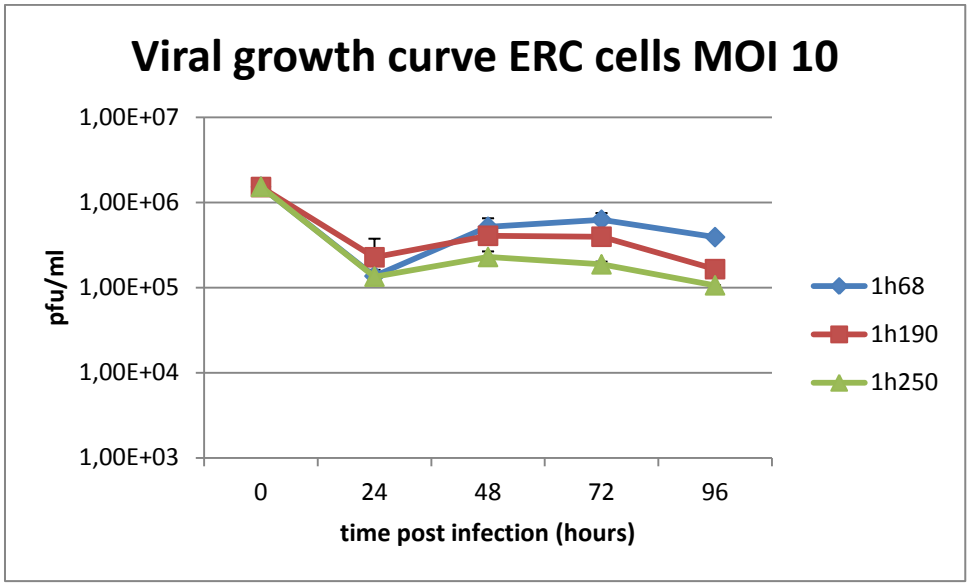


Figure 6.18.: Effect of endogenous microRNA let-7a on rVACV replication

Growth curve in ERC cells incubated with a high MOI of 10. GLV-1h250. The cells were titrated after their harvest.

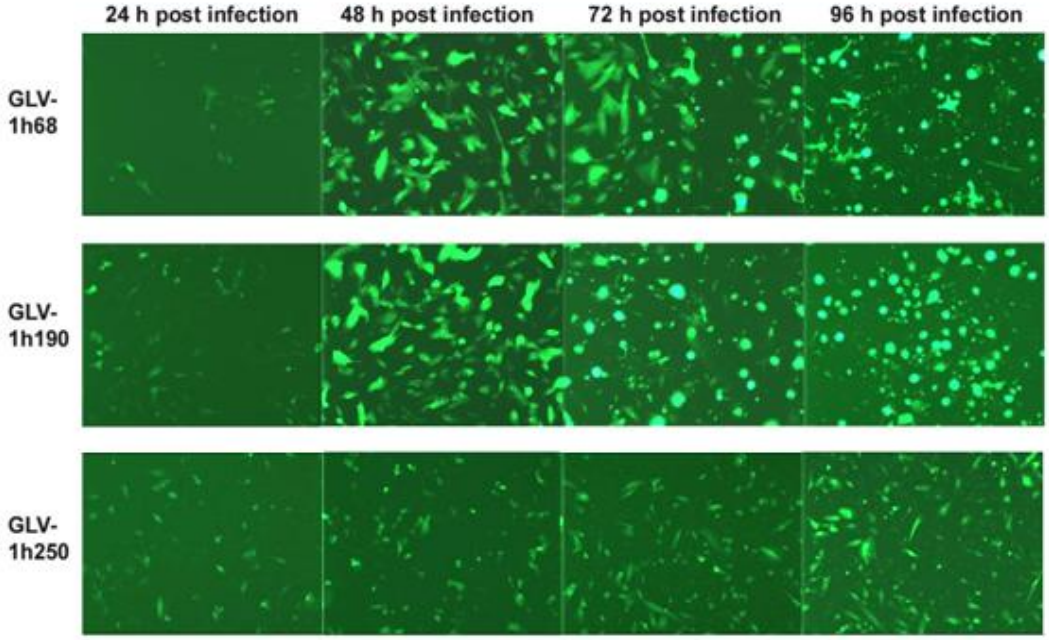


Figure 6.19.: Fluorescence microscopy of GFP expression in ERC cells incubated with virus over 96 hours

gfp expression in ERC cells 24h, 48h, 72h and 96 hours post incubation with virus (MOI 0.01) captured using the UV light source a fluorescent microscope. Viral replication is visible via gfp expression and cell death is displayed by rounded cells.

To investigate whether the oncolytic activity of GLV-1h250 in cancer cells was comparable to GLV-1h68 used in clinical trials (GL-ONC1) studies were conducted

using A549 lung carcinoma cells, a cell line known to show a strong downregulation of microRNA-let-7a. In this case GLV-1h250 showed no significant inhibition of replication when compared to its parental strain and GLV-1h68 at a low MOI of 0.01 over a time course of 96 hours (figure 6.20). These effects could be observed by fluorescence microscopy where marker gene expression was monitored and titration of the harvested cells to investigate the viral load within the cells which leads to the conclusion that the oncolytic activity of GLV-1h250 in cancer cells is not impaired.

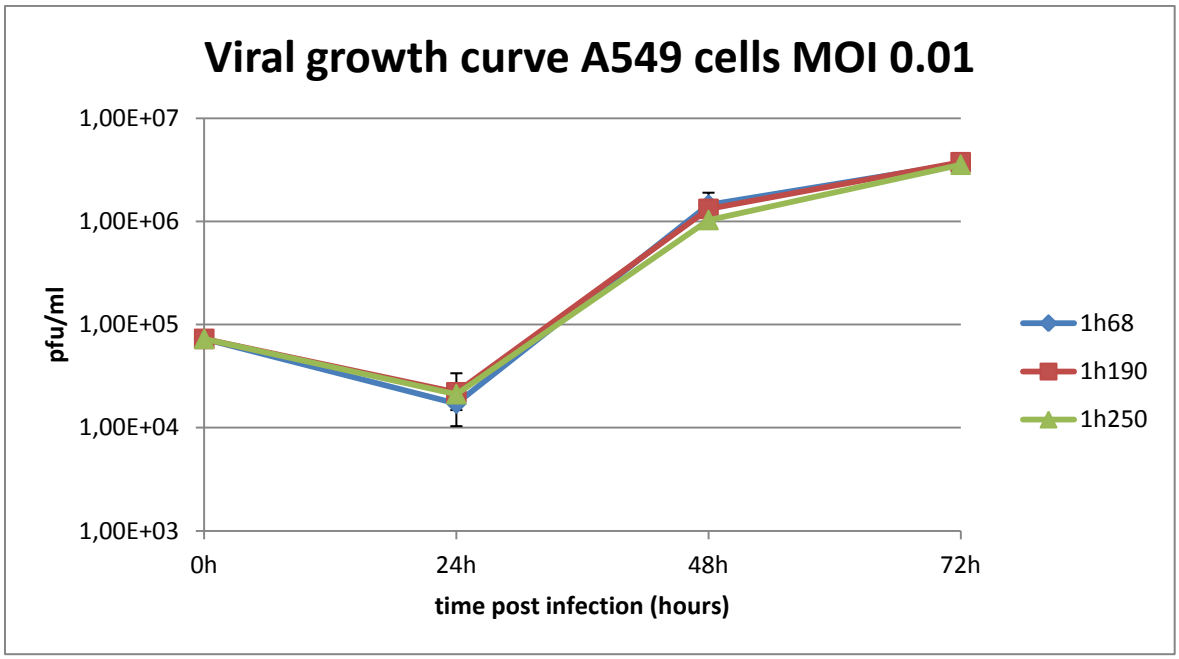


Figure 6.20.: Viral replication in A549 lung adenocarcinoma cells

A low MOI of 0.01 was used in replication studies with A549 cells. The cells were titrated after harvest.

GLV-1h250 showed decreased cytotoxicity displayed by impaired viral replication in human endometrial regenerative cells whereas in cancer cells like the human adenocarcinoma cell line A549 the virus showed comparable oncolytic activity in relation to the viruses GLV-1h68 and GLV-1h190 which displayed strong replication in all tested cells, healthy and cancerous, emphasizing the high specificity and selectivity of GLV-1h250 for replicating in cancer cells rather than healthy cells.

6.4 Oncolytic activity of GLV-1h250 *in vivo*

Toxicity and antitumor efficacy of the newly generated virus GLV-1h250 were investigated *in vivo*. Therefore, A549 lung carcinoma cells were implanted subcutaneous into the right flank of male athymic nude mice. The animals were subsequently injected retro orbitally with high dose of viral particles ($1x10^7$ particle forming units per mouse in 100 µl PBS). A small amount of virus was prepared additionally to what was needed for the injections to confirm the prepared viruses' titers by performing a plaque assay. The behavior of GLV-1h250 *in vivo* was compared to its parental strain GLV-1h190, GLV-1h68 (the strain already used in clinical trials for human application) as a positive control and PBS injected mice as negative controls. Each of the four treatment groups consisted of 15 animals where 5 animals were sacrificed at one week, four weeks and eight weeks post viral injection (overview see figure 6.21). Viral replication could be monitored by observation of gfp expression under a UV light source and could be validated for all participating animals except the PBS treated mice (figure 6.22).

Aim of the study: **Determination of toxicity and oncolytic potential of GLV-1h250 (GLV-1h190 derivative) in comparison to GLV-1h190 with PBS as negative control and GLV-1h68 as positive control in A549 lung adenocarcinoma**

Animal model: **Male athymic nude mice (total: 60 mice)**
Tumor type: **Human A549 lung adenocarcinoma model (5x 10^6 cells/mouse)**
Tumor location: **Subcutaneous right flank**
Viruses: **GLV-1h68, GLV-1h190, GLV-1h250**
Viral dose **1x10^7 pfu/mouse**
Viral delivery route: **retro-orbital**

Summary:

1) Grow tumor subcutaneous in right flank until > 200 mm³
2) Deliver 1x10^7 pfu virus per mouse on retro-orbital route
3) Follow cytotoxicity and anti-tumor efficacy with biweekly tumor volume and body weight measurements, examine overall health status
4) 5 mice per group are sacrificed per week to determine viral load in tumors and organs

Time	Day -0	Day-14	Day-21	Day-28	Day-42
	Implant	Viral Challenge	Titration	Titration	Titration
Group 1 (n=20)	A549	1h190	n=5	n=5	n=5
Group 2 (n=20)	A549	1h250	n=5	n=5	n=5
Group 3 (n=20)	A549	1h68	n=5	n=5	n=5
Group 4 (n=20)	A549	PBS	n=5	n=5	n=5

Figure 6.21.: Overview of GL10-41

The study plan for *in vivo* study GL10-41 for evaluation of the oncolytic efficacy and toxicity of the newly generated virus GLV-1h250 in A549 lung adenocarcinoma bearing male athymic nude mice.

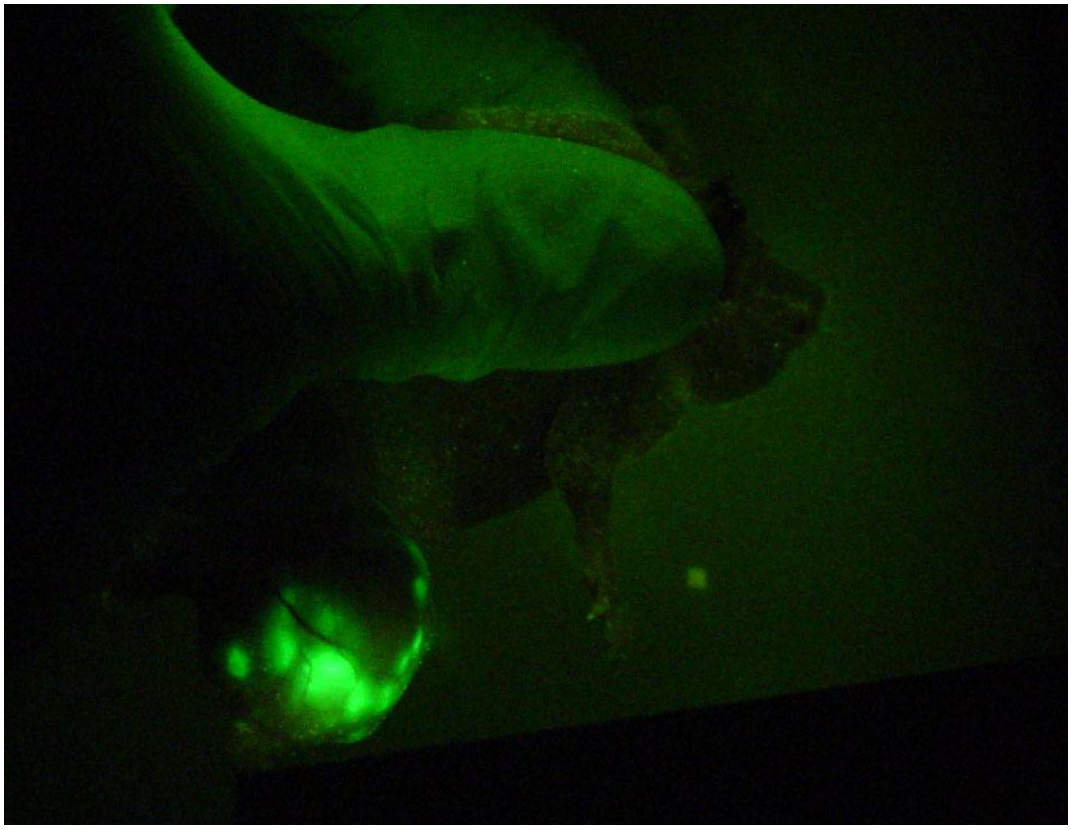


Fig 4622.: gfp expression in A549 bearing mice

Visualization of gfp expression in an A549 lung adenocarcinoma bearing athymic male nude mouse on day 7 after retro-orbital injection with GLV-1h250 under a UV light source.

The antitumor efficacy was determined by measuring the tumors with a caliper twice a week. The toxicity was determined by body weight measurements and examination of the animals for possible pox formation and overall health status. The body weight was stable for most animals although the group treated with GLV-1h250 held the mice with the highest average body weight with tumor volumes comparable to the GLV-1h68 and GLV-1h190 groups (figure 6.23).

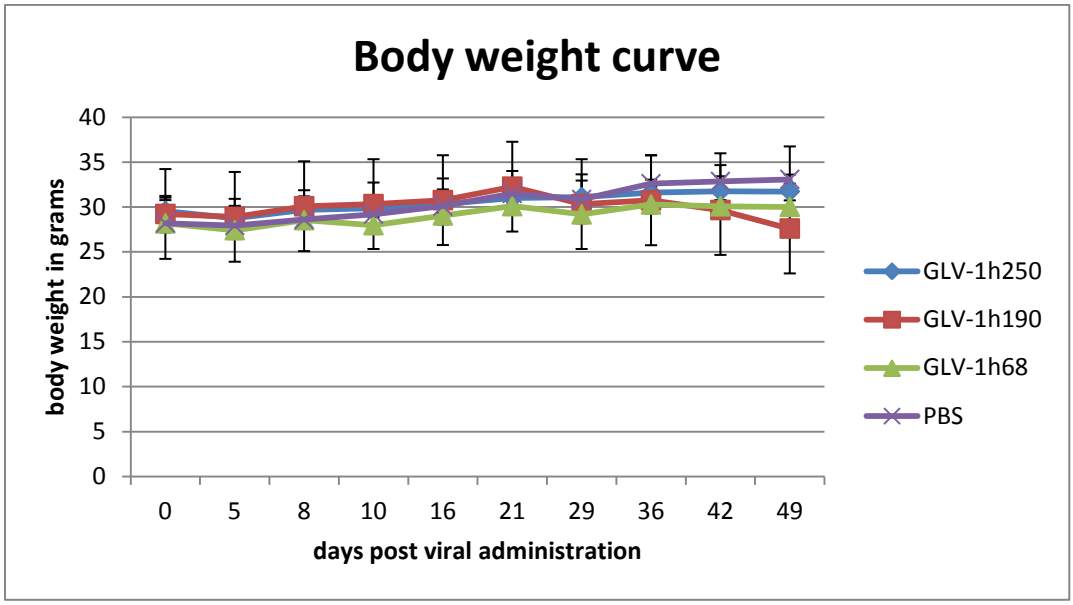


Figure 6.23.: Body weight measurements

Body weight measurements over the course of the study. T-Test significance $p > 0.05$.

The mice treated with either GLV-1h68 or GLV-1h190 displayed animals with toxic side effects in form of pox formation on the body and tail (figures 6.27. A-D, this picture is on the last page of the results part for better comparison of the different groups). Viral toxicity was further accessed by viral biodistribution in various organs. After dissection of the tumors and organs (spleen, liver, lungs, kidneys, testis, brains), they were weighed and placed in MagNA Lyser Green Beads tubes (Roche) containing PBS with proteinase inhibitors. After disruption of the tissues CV-1 cells were incubated with 10 µl of organ lysate. Tumors were diluted until 10^{-6} and the dilutions 10^{-4} – 10^{-6} were used for titration. After two days the cells were stained with crystal violet solution. After washing and drying the virus plaques were counted and titers were calculated. No viral plaques could be found in any of the healthy organs in all mice treated with GLV-1h250 during the complete time course of the study in contrast to the other two tested strains, GLV.1h190 and GLV-1h68. Viral distribution of GLV-1h250 was only present within the tumor (figures 6.24 and 6.25).

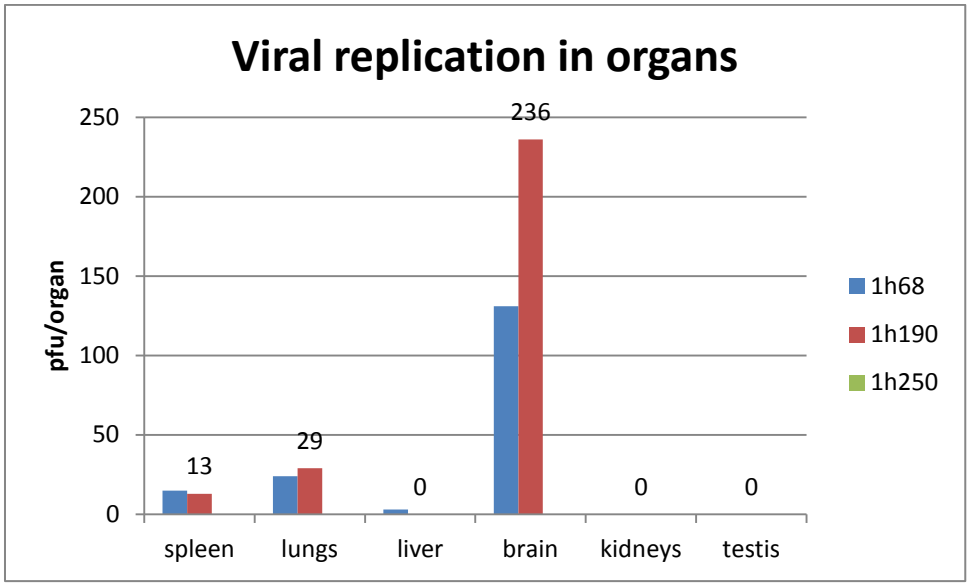


Figure 6.24.: Viral distribution within the body

Viral plaques counted within organs of sacrificed mice participated in this *in vivo* toxicity study. The numbers over the bars represent the averaged virus pfu/organ on day 49 of the study.

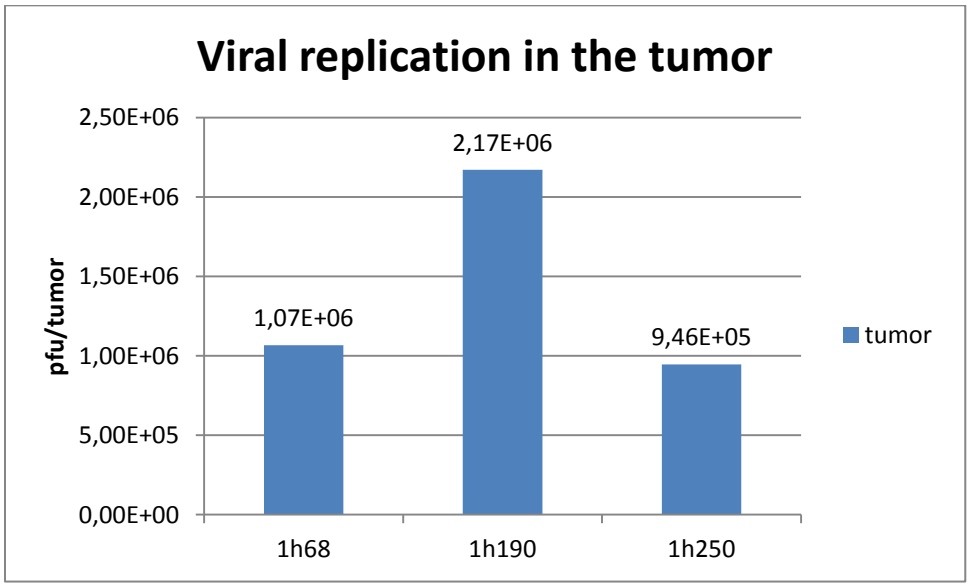


Figure 6.25.: Viral replication within the tumor

Viral replication within the tumor demonstrated as pfu/tumor. GLV-1h250 displayed solid oncolytic potential with reduced toxicity showing no replication potential in healthy cells and tissues as demonstrated *in vitro* and *in vivo* in comparison to GLV-1h190 and GLV-1h68. The numbers over the bars represent the averaged virus pfu/tumor on day 49 of the study.

The oncolytic efficacy of GLV-1h250 showed no significant difference to GLV-1h190 and GLV-1h68 as demonstrated by measured tumor volumes (figure 6.26.)

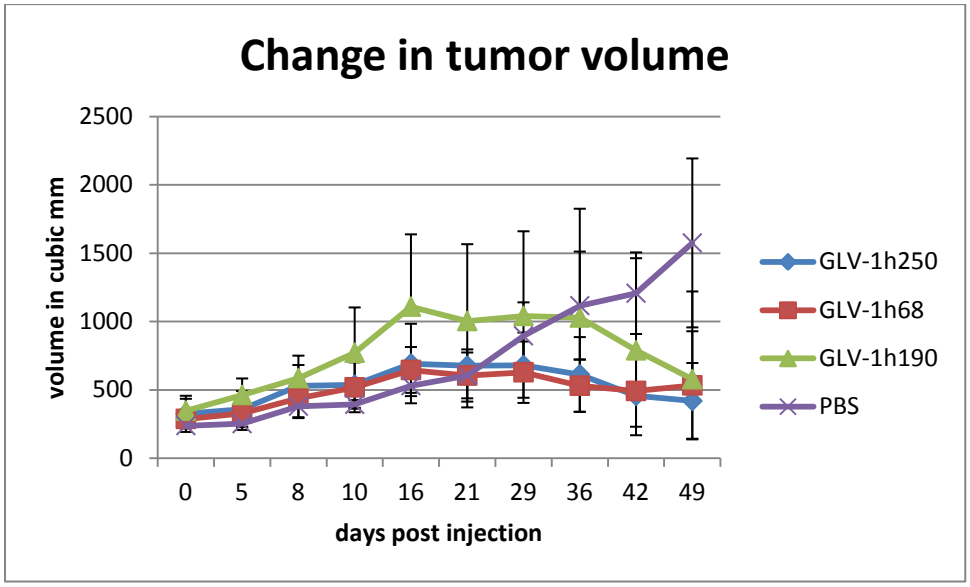
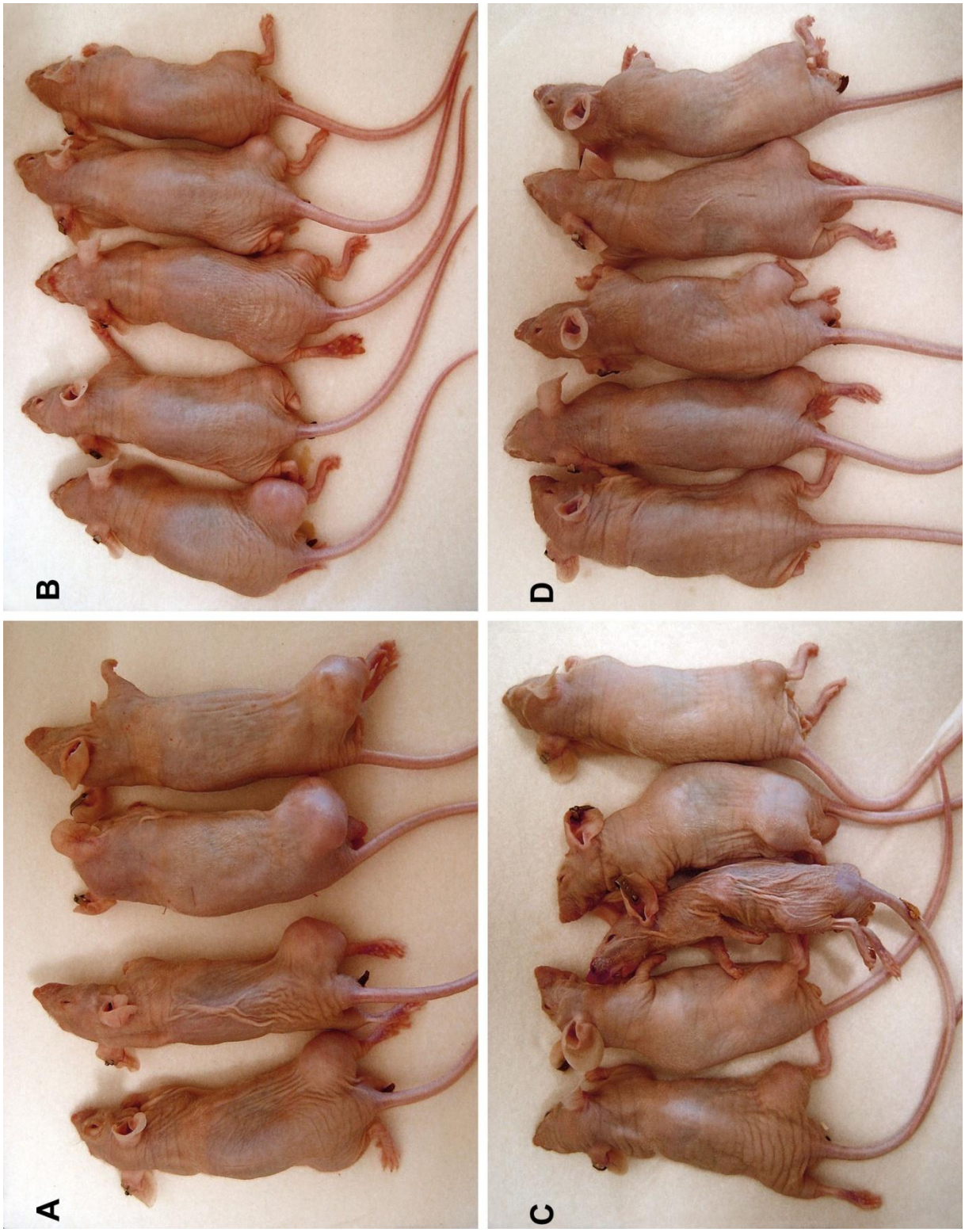


Figure 6.26.: Change in tumor volume

Representation of oncolytic activity of the viruses during the course of the *in vivo* study.

T-Test significance: GLV-1h250 / PBS < 0.05 (only siginificance)



Figures 6.27.: A representation of the general health status of the mice which participated in this in vivo study

(A) the PBS group show solid tumor formation on the right flank; (B) the GLV-1h68 group shows one mouse (middle) displaying pox formation on the left rear paw; (C) the GLV-1h250 group shows no pox formation; (D) mice treated with GLV-1h190 showed pox formation on tail (left and middle) and snout (middle), as well as ears (2nd from right).

GLV-1h250 displayed a strong oncolytic potential with activity comparable to GLV-1h68 whereas viral replication took place solely in the tumor in contrast to its parental strain GLV-1h190 and GLV-1h68.

7 Discussion

Oncolytic virotherapy is a promising approach for the treatment of various types of cancer and has proven to be efficient in clinical trials (Liu et al. 2007). However, novel strategies to suppress undesirable characteristics of these viruses are needed to improve current oncolytic viral specificity and may enable the use of viruses previously considered unsuitable for oncolytic viral cancer therapies. Replication-competent oncolytic vaccinia viruses such as GLV-1h68 are designed for targeted killing of cancer (Yu et al. 2004, Zhang et al. 2009). Other examples of oncolytic virotherapeutics include herpes simplex virus, Newcastle disease virus, adenovirus, vesicular stomatitis virus, myxoma virus, lentivirus and reovirus (Liu et al. 2007). These viruses have deletions of specific virulence genes without which they cannot replicate in healthy cells. Cancer cells often have upregulated homologs of the same deleted genes and therefore create the ability for these attenuated viruses to efficiently replicate in malignant cells. In addition, many oncolytic viruses carry transgenes to allow for assaying or tracking of the virus. The insertion of transgenes into the viral genome can further reduce the ability of oncolytic viruses to productively replicate in healthy cells. Although many potential oncolytic viruses could be designed using these principles, vaccinia virus possesses some distinct advantages. Vaccinia viruses demonstrate an acceptable safety profile in humans as they have been administered to millions in the smallpox vaccine and were studied intensively over many decades. Recombinant oncolytic vaccinia virus GLV-1h68 demonstrates a natural tumor tropism making it significantly safer than other viruses used for an oncolytic approach to target cancer. Yu et al. (2004) observed that tumor bearing

nude mice injected with a recombinant vaccinia virus carrying a light-emitting *Renilla* luciferase-gfp expression cassette displayed green fluorescent patches within the tumor center and periphery at the leaky terminals of the tumor-associated capillary vessels. These results were verified in luciferase reporter assays in which no light emission could be observed outside the tumorous tissues. Additionally, organs, tumors and tumor surrounding muscles and skin were examined and tested for viral plaque growth which was detected exclusively in the tumor. A very important aspect in the targeting of cancer is the recruitment of the innate immune system. Vaccinia virus is highly immunogenic by nature and shows the ability to trigger strong host immune responses against virus-infected cells (Thorne et al. 2005, Woo et al. 2006) creating the ability for the innate immune system to recognize and target the cancer cell which under normal circumstances would remain undetected. Another characteristic of vaccinia virus which makes it a particularly good candidate as an oncolytic vector is its large genome, which is able to carry and express multiple transgenes without an effect on the replicative ability of the virus (McFadden et al. 2005). JX-963, a recombinant vaccinia virus derived from the Western Reserve (WR) strain, was successfully used for tumor targeting. This virus has deletions of viral thymidine kinase (TK) and vaccinia growth factor (VGF) and expresses human GM-CSF (Thorne et al. 2005). JX-963 was shown to be effective against a variety of cancer types (including breast, lung, pancreas, colon, prostate and ovarian cancer cell lines). Thorne et al. (2005) demonstrated antitumor efficacy with this virus in both xenograft and immune competent animal models with both intratumoral and systemic delivery underlining the potential of vaccinia virus in cancer therapy. In addition to therapeutic genes, marker genes can be inserted into oncolytic vectors. Viruses carrying marker genes which are not expressed in cells can be localized and

therefore monitored during the course of viral therapy. Recombinant vaccinia virus GLV-1h68 is a replication-competent virus derived from the vaccinia virus LIVP strain (Lister strain, Institute for Research on Virus Preparations, Moscow, Russia), the construction was previously described (Zhang et al. 2007). This virus carries three insertion cassettes in its genome: a *Renilla* luciferase-green fluorescent protein (RUC-gfp) fusion cassette at the *F14.5L* locus, a reverse inserted human transferrin receptor and β-galactosidase cassette at the *J2R* locus (*J2R* encodes thymidine kinase) and a β-glucuronidase cassette at the *A56R* locus (*A56R* encodes hemagglutinin). The insertions into the three loci decrease the virulence of GLV-1h68 and at the same time offer a possibility for tracking of the virus through marker gene expression of luciferase, gfp, β-galactosidase and β-glucuronidase during its replication in cells. Each one of these marker genes can be replaced to create a new virus with a predictable toxicity and expressing the gene of choice. The vaccinia virus construct GLV-1h190 was derived from GLV-1h68. GLV-1h190 has an insertion of the katushka gene which encodes the TurboFP635 protein, a red fluorescent protein used for deep tissue imaging (Shcherbo et al. 2007). GLV-1h190 displays green and red fluorescent signals and strong oncolytic abilities with an elevated toxicity observed *in vitro* and *in vivo* (data not shown) and was therefore chosen as parental strain for the generation of GLV-1h250. Since this virus has an insertion of four complementary microRNA let-7a repeats in close proximity to the vital vaccinia viral gene *D4R* (*D4R* encodes uracil DNA glycosylase), its replication should be significantly decreased when exposed to microRNA let-7a in a cell. In the absence of microRNA let-7a GLV-1h250s replication should remain strong. GLV-1h250 was able to productively replicate in and lyse human lung adenocarcinoma cells *in vitro* and *in vivo*, thereby displaying no toxic side effects.

As mentioned, recombinant oncolytic viruses are vectors engineered to kill cancer cells while leaving healthy cells unharmed (Parato et al. 2005) and demonstrated positive interaction with chemotherapy (Alonso et al. 2007), radiotherapy (Hingorani et al. 2007) and immunotherapy (Qiao et al. 2008). Clinical data show that this class of therapeutics is safe and cancer selective (Liu et al. 2007). However, as their potency increases, toxicity may as well and improved efficacy of more potent viruses is desirable. Thus, there is a need for more stringency in the regulation of the virus tropism. Various strategies have been used to engineer oncolytic viruses to limit their replication to cancer cells (Parato et al. 2005). Viral genes required for countering host cell innate immunity are deleted or mutated in a variety of oncolytics. The approach of employing deletions or mutations in viral genes that have evolved to counter the host antiviral response is a common strategy to design safe oncolytic viruses (Muster et al. 2004, Hummel et al. 2005). This strategy has been used with oncolytic vesicular stomatitis virus (Stojdl et al. 2000), influenza (Muster et al. 2004), and herpes simplex virus-1 (Varghese et al. 2002). Increasing evidence for an association between microRNA expression and cancer (Calin et al. 2006, Cummins et al. 2006) along with the number of examples of endogenous cellular microRNAs with effective antiviral activity (Pedersen et al. 2007, Lecellier et al. 2005) led to the hypothesis that exploiting microRNA expression could be an efficient strategy to direct replication of therapeutic viruses to target tissues and has proven to be successful in a variety of generated oncolytic viruses using microRNA for tumor targeting (Liu et al. 2007, Cawood et al. 2009, Corell et al. 2011). This principle was developed by Brown et al. (2006), who showed that insertion of microRNA 142-3p binding sites in the 3'-UTR (untranslated region) of retroviral encoded transgenes disabled expression in antigen presenting cells, thereby preventing stimulation of an

immune response and allowing long term transgene expression in other cells without rejection. Unlike the redirection of viral infectivity to the tumor cell surface, termed transductional targeting, strategies which might increase the pathogenicity of viruses by increasing their host range, microRNA-mediated regulation is highly unlikely to do so, as it restricts host range by using pre-existing cellular microRNAs to act as a primed antiviral defense mechanism. Many potent virotherapeutics are associated with well documented toxicities to the brain, such as poliovirus, herpes simplex virus, vesicular stomatitis virus, Egypt 101 virus and West Nile virus (Kelly et al. 2007, Barber et al. 2004). Toxicity to the heart was associated with reovirus (Qiao et al. 2008), adenoviruses showed toxicity to the liver (Heise et al. 1999). Therefore, research was conducted in which tissue-specific microRNA targets were incorporated into these viruses to increase their therapeutic relevance without decreasing their antitumor activity. Lee et al. 2009 showed that in PC3 prostate cancer cells endogenous microRNAs could be exploited to regulate the expression of an essential herpes simplex virus-1 viral gene in a sequence-specific manner, resulting in viruses that were selectively oncolytic for tumor cells while leaving healthy tissues unharmed. The finding that oncolytic vaccinia viruses were sensitive to microRNA-mediated repression (Hikishi et al. 2011) combined with the advantages of recombinant vaccinia virus GLV-1h68 as an oncolytic agent suggested a potential success when utilizing the concept of exploiting endogenous microRNA expression levels in healthy cells to limit viral replication to cancer cells.

MicroRNAs are noncoding RNAs of approximately 22 nucleotides in length which mediate posttranscriptional regulation through specific recognition of short sequences which are mostly located in the 3`-UTR of the target mRNAs (Ivanovska et al. 2008).

Depending on the degree of complementarity to the target, the microRNA can affect either the stability or translation of the mRNA (Zeng et al. 2003). MicroRNAs display complex expression profiles reflecting the importance of their role in the control of cell growth and development. The human genome is estimated to contain more than 500 microRNA genes. Some genes are expressed in a tissue-specific manner whereas others are constitutively expressed or activated in response to endogenous signals or stress (Xu et al. 2007). Another important function of microRNAs, clearly demonstrated in plants and invertebrates (Obbard et al. 2008), is to suppress viruses by binding to cognate sequences in viral mRNAs. The let-7 family of microRNAs is highly conserved and found ubiquitously expressed in mammalian cells (Sempere et al. 2004, Lagos-Quintana et al. 2003, Pasquinelli et al. 2000). The expression of let-7a is associated with differentiation and is expressed at low levels in cancer cells (Jannot et al. 2006, Karube et al. 2006, Kent et al. 2006, Takamizawa et al. 2004, Yanaihara et al. 2006, Viswanathan et al. 2008) and is downregulated in less differentiated breast tumor initiator cells (Yu F. et al. 2007). In addition to tumor specific low expression, let-7a was thought to be suitable to act as replication control due to its high expression and activity in the majority of differentiated healthy cells. High expression may be required to compete with a rapidly replicating lytic virus such as vaccinia virus whereas microRNA expression in most non-target tissues may be required to eliminate pathogenic replication *in vivo*. Let-7a has these properties and was suitable for this particular method of targeting cancer cells with an engineered oncolytic. On the other hand, with many microRNAs serving a role as tumor suppressors (Kent et al. 2006, He et al. 2007), other microRNA target sites or combinations of microRNA target sites may be useful in optimizing vaccinia virus mediated oncolysis.

Although there exists no specific microRNA expression profile common to cancerous cells, such expression profiles reflect the developmental linage and differentiation state of tumors (Lu et al. 2005). This might be relevant due to a potential diagnostic value of microRNA expression profiles and direct an oncolytic therapeutic strategy such as presented in this thesis. The incorporation of microRNA target sites into oncolytic vaccinia virus should result in a virus that is attenuated in healthy cells and has reduced *in vivo* toxicity while retaining antitumor activity, a necessity to control the replication of more potent viruses. Coxsackievirus A21 (CVA21) is a naturally oncolytic virus which displays strong replication in various types of cancer cells (Berry et al. 2008). However, CVA21 also induces pathology in mice and humans by replicating in healthy muscle cells (Dekel et al. 2002). Kelly et al. (2008) showed that by incorporating four microRNA target sequences into the CVA21 genome, they could reduce viral replication in healthy muscle tissue by more than 100,000-fold and thereby eliminate muscle pathology without compromising tumor-killing activity. In addition, Ylösmäki et al. (2008) created an adenovirus regulated by miR122, a liver-specific microRNA, to reduce toxic side effects. An early gene product of adenovirus (E1A) was tagged with the target sequence for miR122 and effectively eliminated the ability of the adenovirus to replicate in healthy liver cells. Aberrant microRNA expression was shown to contribute to the generation of malignancies (Deng et al. 2008) and more 'tumor-suppressor' microRNAs are discovered to be ubiquitously expressed in healthy tissues but often reduced or absent in malignant cells (He et al. 2007). Edge et al. (2008) demonstrated that incorporating the target sequence for the let-7a tumor-suppressor microRNA into the genome of vesicular stomatitis virus generated a virus that replicates preferentially in tumor cells. The let-7a microRNA is often expressed at lower levels in tumors than in healthy tissues serving as natural

replication control for recombinant viruses such as GLV-1h250. GLV-1h250 displayed a 10-fold reduction in replication rates in human endometrial replicative cells (ERC) when compared to its parental strain GLV-1h190 and GLV-1h68 suggesting a microRNA let-7a dependent ability of GLV-1h250 to replicate in cells. ERC are considered non-cancerous cells and therefore thought to display a microRNA expression pattern with a high endogenous level of microRNA let-7a. In contrast, when A549 cells were incubated with GLV-1h250, GLV-1h190 and GLV-1h68, all three viruses showed similar oncolytic abilities verified by comparison of marker gene expression and viral titers. These results lead to the conclusion that replication of recombinant vaccinia virus GLV-1h250 can indeed be limited to cancer cells due to endogenous microRNA levels. However, quantitative real-time PCR experiments are necessary to verify a downregulation of the GLV-1h250 mRNA after virus uptake and replication in healthy cells to prove the let-7a dependent degradation of the viral mRNA on the transcriptional level. Additionally, the levels of endogenous microRNA let-7a should be controlled to investigate if the presence of multiple target structures following the incubation with GLV-1h250 has any impact on cellular microRNA let-7a levels. A decrease of endogenous microRNA let-7a levels could act counterproductive and cause alterations in concentrations of oncogenes such as RAS or C-MYC, proteins which are regulated by let-7a and play an important role in cell proliferation and division. The observed decrease in viral replication in healthy cells needs further verification on the translational level as the conducted protein gel and Western blot did not yield obvious results. After staining the gel with coomassie blue, bands of the size expected for the D4R protein (25 kD) were visible for all samples including the control suggesting the staining of other proteins but the target. The subsequently performed Western blot yielded weak bands of expected size in all

samples suggesting unspecific binding of the anti-D4R antibody. This could be due to the construction of the anti-D4R-antibody used for detection as it might have been designed against an epitope which might not accessible during the western blot analysis thereby leading to unspecific binding and an unclear result. A successful inhibition of vaccinia virus D4R protein generation mediated by endogenous microRNA let-7a levels would be characterized by an overall decrease in viral protein levels. Since the D4R uracil glycosylase is essential for DNA replication in vaccinia virus (Stuart et al. 1993) the viral replication should significantly decrease after exposure of the target structure on the viral mRNA to cellular microRNA let-7a. This should be detectable shortly after the virus uptake and replication in cells high in microRNA let-7a expression due to the early expression of the D4R gene in vaccinia virus life cycle. *In vivo*, GLV-1h250 showed the ability to regress subcutaneously grown A549 lung adenocarcinomas at a rate comparable with GLV-1h68 and GLV-1h190 although the size and concentration of the viral particles analyzed after dissection of the animals was slightly decreased in relation to the other two viruses. However, at the time the experiment was terminated all animals treated with GLV-1h250 showed a significant reduction in tumor size suggesting a successful therapeutic potential. Most importantly, although GLV-1h250 displayed successful treatment of subcutaneous A549 tumors, it did not result in any signs of toxicity or replication in healthy host tissues over a varied time period, from 24 hr to 7 weeks after viral administration in contrast to GLV-1h68 and GLV-1h190. After retro orbital administration these two viruses showed viral particles in various organs analyzed *post mortem* like the spleen, the lungs or the brain. Viral particles were found from 2 weeks after administration of the viruses throughout the length of the study. Antitumor therapy with GLV-1h250 was well tolerated by athymic mice and resulted

in no signs of toxicity and displayed no replicative ability in healthy tissues. A limitation of this model is the fact that these mice are immune compromised. It would have to be investigated whether the immune system might significantly decrease viral efficacy due to the observed delay in viral growth or if the virus might be cleared from the body by an intact immune system.

Gene silencing by endogenous microRNAs can be exploited to control the tropism of gene-therapy vectors. Brown et al. (2006, 2007) suppressed transgene expression specifically in hematopoietic cells and hepatocytes by including the target sequence of a tissue-specific microRNA in the genome of lentiviral vectors, a strategy subsequently extended to control targeting of other oncolytic viruses to yield safer and more effective anticancer virotherapeutics. Despite the lack of transcriptional and translational data, when administered *in vivo* the replication of GLV-1h250 took place solely in the tumor suggesting efficient replication control outside the cancer cell whereas its parental strain and GLV-1h68 both showed viral colonization of healthy tissues. So far, the research on oncolytic viruses has explored various ways of restricting viral replication to malignant tissues such as engineering virus coat proteins to recognize unique tumor antigens on the cell surface, transcriptionally regulating viral genes and exploiting activated signaling pathways unique to tumor cells (Parato et al. 2005). However, microRNA targeting has several features that may ultimately make it one of the preferred methods for engineering viral tropism, either alone or in combination with complementary approaches. For instance, the microRNA sequences required to regulate virus replication are short and portable, making them easy to add even to smaller viral genomes. In contrast, transductional targeting requires severe alterations of the viral genome that often attenuate viral

growth even in target cells. Another advantage is that the strategy works for both, RNA and DNA viruses, and whether they replicate in the cytoplasm or in the nucleus (Brown et al. 2006 and 2007, Edge et al. 2008, Ylösmäki et al. 2008, Kelly et al. 2008). In contrast, transcriptional targeting of oncolytic viruses can be used only for DNA viruses that replicate in the nucleus. As Kelly et al. (2008) demonstrated, regulating oncolytic virus replication with preexisting highly expressed cellular microRNAs could provide a more potent defense without the necessity to be primed by viral challenge. The use of tissue-specific microRNAs could eliminate the strong toxicity observed in many oncolytic viruses. However, previously unrecognized secondary complications have to be considered. For instance, Kelly et al. (2008) found that although their microRNA-targeted adenovirus no longer caused acute muscle myositis, myelitis associated with tremors and paralysis was observed in a small number of animals. This may have been due to the extended viremia caused by the engineered virus. The group suggested that the incorporation of a tissue-specific virus 'silencer' together with a more ubiquitous tumor-suppressor microRNA target could solve this problem. GLV-1h250 was ultimately derived from GLV-1h68, an oncolytic vaccinia virus prototype with little but present toxicity *in vivo*. GLV-1h250 showed no signs of toxicity when administered in mice and the replication took place exclusively in the tumor. This suggests that the insertion of the microRNA let-7a complementary sites further increased the tropism of GLV-1h250 towards cancer cells leaving no possibility to replicate in healthy tissues *in vivo*. Another issue concerns the number of microRNA targets to use and where to insert them. In recent studies (Edge et al. 2008, Ylösmäki et al. 2008, Kelly et al. 2008), multiple copies of microRNA targets were placed in the 3′ UTRs of viral genes. However, the optimal site might be specific to the generated virus and might need further investigation.

Barnes et al. (2008) created an attenuated poliovirus vaccine strain by including one microRNA target sequence in the 5′ UTR and a second one between two coding regions. The attenuated strain was unable to replicate in the central nervous system, thus eliciting protective immunity without pathological side effects. Reports indicated that four tandem-repeat copies of a single target element are most efficacious for the silencing of gene expression (Brown et al. 2007). This approach was considered in the generation of the recombinant vaccinia virus GLV-1h250. Four complementary microRNA let-7a repeats were inserted at the 3´-UTR of the D4R gene followed by the stop codon resulting in a ten-fold decrease in viral replication when incubated with human endometrial regenerative cells in relation to the parental virus GLV-1h190 and GLV-1h68 suggesting a microRNA let-7a dependent replication.

Engineered oncolytic viruses carrying genomic deletions in viral genes to increase safety could instead have these genes under the regulatory control of incorporated microRNA target sites. Examples of oncolytic viruses and the corresponding viral gene deleted in current oncolytics that may be suitable for this approach include HSV-1 and the γ 34.5 gene, influenza virus and the NS-1 gene, and vesicular stomatitis virus's M protein (Parato et al. 2005, Stojdl et al. 2003). Some viruses, such as picornaviruses may also be suitable for microRNA-mediated regulation of replication. However, they have demonstrated an ability to produce escape mutants when engineered to carry a let-7 microRNA target site (Lu et al. 2005, Gitlin et al. 2005) although the majority would be eliminated by innate or adaptive immune responses (Edge et al. 2008, Kelly et al. 2008, Barnes et al. 2008). The generation of escape mutants was found to be triggered by single mismatches within the target sequence of the microRNA. These aspects were considered in the generation of

GLV-1h250 and an insertion of four let-7a complementary repeats was used to avoid this phenomenon further emphasizing Kelly´s et al. (2008) suggestion that it may be necessary for oncolytic viruses to carry multiple repeats or repeats of different microRNA target sites to prevent the production of escape mutants. As the complexities of microRNA expression and function are elucidated, many opportunities to improve the design of oncolytic viruses will emerge. When used in combination with microRNA profiling of a patient's tumor (Kong et al. 2008), these viruses could provide a so far unreached level of personalized medicine and enhance clinical outcomes for cancer patients.

A microRNA-mediated restriction provides a reliable method to generate selective impotency of a pathogen to replicate in its tissue of choice and provides a new approach for generating *de novo* oncolytic viruses and improving the safety of the ones already in use. GLV-1h250 was able to maintain the successful oncolytic effect achieved against A549 lung adenocarcinoma cells *in vitro* and in an orthotopic animal model of A549 adenocarcinoma *in vivo* when compared to its parental strain GLV-1h190 and GLV-1h68. GLV-1h250 successfully localized, replicated, and displayed detectable marker gene expression in tumor tissues after retro orbital administration. Animals treated with GLV-1 h 250 maintained healthy weights and displayed no signs of toxicity throughout the entire treatment whereas control animals treated with the parental strain GLV-1h190 showed pox formation on various body parts along with a decrease in body weight and overall health starting 2-3 weeks after inoculation. This supports the data obtained when conducting the growth curves in A549 tumor cells and ERC cells *in vitro* while comparing the replication efficacy of GLV-1h250 with GLV-1h190 and GLV-1h68. GLV-1h250 efficiently distinguished between healthy and

cancer cells by showing a ten-fold reduction in ERC cells and strong replication rates in A549 cells suggesting efficient oncolytic potential while displaying effective control by endogenous microRNA let-7a levels. MicroRNAs are emerging as new potent and active regulators of cellular gene expression. This thesis demonstrates that naturally occurring and differentially expressed microRNA let-7a can be exploited to control vaccinia virus replication. Overall, the data provide a proof of principle that the life cycles of replication-competent vaccinia virus GLV-1h250 can be modified to direct tropism towards cancer cells and therefore enhance safety of oncolytic vaccinia virotherapeutics already in use and provide a new paradigm for the attenuation of pathogens and the creation of safer products for targeted cancer therapy. GLV-1h250 successfully demonstrated comparable oncolytic activity while displaying no toxicity when applied for cancer treatment in mice suggesting a replication dependent on endogenous microRNA let-7a levels. However, additional *in vivo* studies are necessary to investigate whether GLV-1h250 can demonstrate oncolytic activity when using a lower viral dose analogous to the dose used in clinical trials with GLV-1h68 to treat human tumors. The observed *in vitro* and *in vivo* results were not and yet have to be verified on the transcriptional and translational level to further prove the effect of the endogenous microRNA-let7a levels on the replication of the virus. Additionally, further investigation of the oncolytic potential of GLV-1h250 using different cancer cell lines will be necessary to confirm its efficacy in other types of cancer cells with alterated let-7a expression levels.

In conclusion, GLV-1h250 demonstrates excellent tumor specificity, replicative ability, and cytotoxic efficacy in A549 lung adenocarcinoma and warrants further investigation as a safe therapeutic agent providing a targeted approach against

cancers with low microRNA let-7a expression levels. The findings in this study suggest that other tissue specific microRNAs may be used for the targeting of vaccinia virus replication and that the same principle may be applied to non-attenuated wild-type viruses which are under principle consideration for the application in oncolytic virotherapy but were found to be too toxic to healthy tissues. Since the application of high viral doses could lead to adverse effects in human patients as demonstrated in various clinical trials with adenoviruses (Harvey et al. 2002, Yen et al. 2000, Raper et al. 2002, Mickelsen 2002) new approaches to further limit viral replication to cancer cells may be crucial to guarantee the secure administration of oncolytic viruses in clinical settings despite the increase in safety of systemically delivered oncolytic viruses in recent years (Kumar et al. 2008, Markert et al. 2000, Park et al. 2008, Vitell-Pedersen et al. 2011). By expressing microRNA target structures in virus genomes, viral replication was shown to successfully be inhibited in tissues expressing cognate microRNAs (Edge et al. 2008, Kelly et al. 2008 and 2010, Lee et al. 2009, Ylösmaki et al. 2008). The appropriation of microRNAs expressed by viral vectors could also be deployed as biomarkers in cancer profiling (Rosenfeld et al. 2008), thereby identifying cancer metastases by their site of origin which could be applied in individuals with cancer of unknown primary source. MicroRNA replacement therapy to alter cancer cell behavior illustrates another potential use of microRNAs in viral vector targeting of cancer, a method shown to be successful for viruses which replicate within the nucleus (Roth et al. 2006, Kota et al. 2009, Fulmer et al. 2010). Vaccinia virus, which exclusively replicates in the cytoplasm, cannot utilize the nuclear splicing machinery to produce mature microRNAs. In this case, self-cleaving enzymes such as ribozymes could be incorporated into the viral genome to replace the nuclear splicing machinery

(Suryawanshi et al. 2010) to yield mature microRNAs. The generation of a microRNA sensitive vaccinia virus could be the first step to achieving the goal of assembling components of the nuclear splicing machinery in the cytoplasm of eukaryotic cells by successful expression and processing of a target mRNA synthesized by vaccinia virus which is recognized by endogenous miRNAs.

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